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(21) International Application Number: PCT/GB89/01343 (22) International Filing Date: 13 November 1989 (13.11.89) (30) Priority data: 8826446.0 11 November 1988 (11.11.88) GB (71) Applicant (for all designated States except US): PHARMA- CEUTICAL PROTEINS LIMITED [GB/GB]; Orchard Brae House, 30 Queensferry Road, Edinburgh EH4 2HG (GB). (72) Inventors; and (75) Inventors/Applicants (for US only) : ARCHIBALD, Alan, Langskill [GB/GB]; 70 Silverknowes View, Edinburgh EH4 5PS (GB). CLARK, Anthony, John [GB/GB]; 29 Broomieknowe, Lasswade, Midlothian EH18 1LN (GB). HARRIS, Stephen [GB/GB]; 3 Leamington Road, Edin- burgh EH3 9PD (GB). McCLENAGHAN, Margaret [GB/GB]; 8 Livingstone Place, Edinburgh EH9 1PA (GB). SIMONS, John, Paul [GB/GB]; 94 Warrender Park Road, Edinburgh EH9 1ET (GB). WHITELOW, Christopher, Bruce, Alexander [GB/GB];	43 Viewforth, Edinburgh EH10 4LA (GB). (74) Agents: SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB). (81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CH (European patent), DE (European pa- tent), DK, FI, FR (European patent), GB (European pa- tent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European pa- tent), SU, US. Published <i>With international search report.</i>	
(54) Title: A GENETIC CONSTRUCT OF WHICH PROTEIN-CODING DNA COMPRISES INTRONS AND IS DE- SIGNATED FOR PROTEIN PRODUCTION IN TRANSGENIC ANIMALS (57) Abstract Proteinaceous products can be produced by transgenic animals having genetic constructs integrated into their genome. The construct comprises a 5'-flanking sequence from a mammalian milk protein gene (such as beta-lactoglobulin) and DNA coding for a heterologous protein other than the milk protein (for example a serin protease such as alpha ₁ -antitrypsin or a blood factor such as Factor VIII or IX). The protein-coding DNA comprises at least one, but not all, of the introns naturally occurring in a gene coding for the heterologous protein. The 5'-flanking sequence is sufficient to drive expression of the heterologous protein.		

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1 A genetic construct of which protein coding DNA comprises
2 introns and is designed for protein production in trans-
3 genic animals.

4 This invention relates to the production of
5 peptide-containing molecules.

6 Recombinant DNA technology has been used increasingly
7 over the past decade for the production of commercially
8 important biological materials. To this end, the DNA
9 sequences encoding a variety of medically important
10 human proteins have been cloned. These include
11 insulin, plasminogen activator, α_1 -antitrypsin and
12 coagulation factors VIII and IX. At present, even with
13 the emergent recombinant DNA techniques, these proteins
14 are usually purified from blood and tissue, an
15 expensive and time consuming process which may carry
16 the risk of transmitting infectious agents such as
17 those causing AIDS and hepatitis.

18
19 Although the expression of DNA sequences in bacteria to
20 produce the desired medically important protein looks
21 an attractive proposition, in practice the bacteria
22 often prove unsatisfactory as hosts because in the
23 bacterial cell foreign proteins are unstable and are
24 not processed correctly.

25
26 Recognising this problem, the expression of cloned
27 genes in mammalian tissue culture has been attempted
28 and has in some instances proved a viable strategy.
29 However batch fermentation of animal cells is an
30 expensive and technically demanding process.

31
32 There is therefore a need for a high yield, low cost
33 process for the production of biological substances

1 such as correctly modified eukaryotic polypeptides.
2 The absence of agents that are infectious to humans
3 would be an advantage in such a process.

4
5 The use of transgenic animals as hosts has been
6 identified as a potential solution to the above
7 problem. WO-A-8800239 discloses transgenic animals
8 which secrete a valuable pharmaceutical protein, in
9 this case Factor IX, into the milk of transgenic sheep.
10 EP-A-0264166 also discloses the general idea of
11 transgenic animals secreting pharmaceutical proteins
12 into their milk, but gives no demonstration that the
13 technique is workable.

14
15 Although the pioneering work disclosed in WO-A-8800239
16 is impressive in its own right, it would be desirable
17 for commercial purposes to improve upon the yields of
18 proteins produced in the milk of the transgenic animal.
19 For Factor IX, for example, expression levels in milk
20 of at least 50 mcg/ml may be commercially highly
21 desirable, and it is possible that for α_1 -
22 antitrypsin higher levels of expression, such as 500
23 mcg/ml or more may be appropriate for getting a
24 suitably high commercial return.

25
26 It would also be desirable if it was possible to
27 improve the reliability of transgenic expression, as
28 well as the quantitative yield of expression. In other
29 words, a reasonable proportion of the initial
30 Generation 0 (G0) transgenic animals, or lines
31 established from them, should express at reasonable
32 levels. The generality of the technique, in
33 particular, is going to be limited if (say) only one in

1 a hundred animals or lines express. This is
2 particularly the case for large animals, for which,
3 with the techniques currently available, much time and
4 money can be expended to produce only a small number of
5 G0 animals.

6
7 Early work with transgenic animals, as represented by
8 WO-A-8800239 has used genetic constructs based on cDNA
9 coding for the protein of interest. The cDNA will be
10 smaller than the natural gene, assuming that the
11 natural gene has introns, and for that reason is more
12 easy to manipulate.

13
14 Brinster et al (PNAS 85 836-840 (1988)) have
15 demonstrated that introns increase the transcriptional
16 efficiency of transgenes in transgenic mice. Brinster
17 et al show that all the exons and introns of a natural
18 gene are important both for efficient and for reliable
19 expression (that is to say, both the levels of the
20 expression and the proportion of expressing animals)
21 and is due to the presence of the natural introns in
22 that gene. It is known that in some cases this is not
23 attributable to the presence of tissue-specific
24 regulatory sequences in introns, because the phenomenon
25 is observed when the expression of a gene is redirected
26 by a heterologous promoter to a tissue in which it is
27 not normally expressed. Brinster et al say that the
28 effect is peculiar to transgenic animals and is not
29 seen in cell lines.

30
31 It might therefore be expected that the way to solve
32 the problems of yield and reliability of expression
33 would be simply to follow the teaching of Brinster et

1 al and to insert into mammalian genomes transgenes
2 based on natural foreign genes as opposed to foreign
3 cDNA. Unfortunately, this approach is itself
4 problematical. First, as mentioned above, natural
5 genes having introns will inevitably be larger than the
6 cDNA coding for the product of the gene. This is
7 simply because the introns are removed from the primary
8 transcription product before export from the nucleus as
9 mRNA. It is technically difficult to handle large
10 genomic DNA. Approximately 20 kb, for example,
11 constitutes the maximum possible cloning size for
12 lambda-phage. The use of other vectors such as
13 cosmids, may increase the handleable size up to 40 kb,
14 but there is then a greater chance of instability. It
15 should be noted that eukaryotic DNA contains repeated
16 DNA sequence elements that can contribute to
17 instability. The larger the piece of DNA the greater
18 the chance that two or more of these elements will
19 occur, and this may promote instability.

20
21 Secondly, even if it is technically possible to
22 manipulate large fragments of genomic DNA, the longer
23 the length of manipulated DNA, the greater chance that
24 restriction sites occur more than once, thereby making
25 manipulation more difficult. This is especially so
26 given the fact that in most transgenic techniques, the
27 DNA to be inserted into the mammalian genome will often
28 be isolated from prokaryotic vector sequences (because
29 the DNA will have been manipulated in a prokaryotic
30 vector, for choice). The prokaryotic vector sequences
31 usually have to be removed, because they tend to
32 inhibit expression. So the longer the piece of DNA,
33 the more difficult it is to find a restriction enzyme
34 which will not cleave it internally.

1 To illustrate this problem, α_1 -antitrypsin, Factor
2 IX and Factor VIII will briefly be considered. α_1 -
3 antitrypsin (AAT) comprises 394 amino acids as a mature
4 peptide. It is initially expressed as a 418 amino acid
5 pre-protein. The mRNA coding for the pre-protein is
6 1.4 kb long, and this corresponds approximately to the
7 length of the cDNA coding for AAT used in the present
8 application (approximately 1.3 kb). The structural
9 gene (liver version, Perlino et al, The EMBO Journal
10 Volume 6 p.2767-2771 (1987)) coding for AAT contains 4
11 introns and is 10.2 kb long.

12
13 Factor IX (FIX) is initially expressed as a 415 amino
14 acid preprotein. The mRNA is 2.8 kb long, and the cDNA
15 that was used in WO-A-8800239 to build FIX constructs
16 was 1.57 kb long. The structural gene is approximately
17 34 kb long and comprises 7 introns.

18
19 Factor VIII (FVIII) is expressed as a 2,351 amino acid
20 preprotein, which is trimmed to a mature protein of
21 2,332 amino acids. The mRNA is 9.0 kb in length,
22 whereas the structural gene is 185 kb long.

23
24 It would therefore be desirable to improve upon the
25 yields and reliability of transgenic techniques
26 obtained when using constructs based on cDNA, but
27 without running into the size difficulties associated
28 with the natural gene together with all its introns.

29
30 It has now been discovered that high yields can be
31 obtained using constructs comprising some but not all,
32 of the naturally occurring introns in a gene.

33

1 According to a first aspect of the present invention,
2 there is provided a genetic construct comprising a 5'
3 flanking sequence from a mammalian milk protein gene
4 and DNA coding for a heterologous protein other than
5 the milk protein, wherein the protein-coding DNA
6 comprises at least one, but not all, of the introns
7 naturally occurring in a gene coding for the
8 heterologous protein and wherein the 5'-flanking
9 sequence is sufficient to drive expression of the
10 heterologous protein.

11

12 The milk protein gene may be the gene for whey acid
13 protein, alpha-lactalbumin or a casein, but the
14 beta-lactoglobulin gene is particularly preferred.

15

16 In this specification the term "intron" includes the
17 whole of any natural intron or part thereof.

18

19 The construct will generally be suitable for use in
20 expressing the heterologous protein in a transgenic
21 animal. Expression may take place in a secretory gland
22 such as the salivary gland or the mammary gland. The
23 mammary gland is preferred.

24

25 The species of animals selected for expression is not
26 particularly critical, and will be selected by those
27 skilled in the art to be suitable for their needs.
28 Clearly, if secretion in the mammary gland is the
29 primary goal, as is the case with preferred embodiments
30 of the invention, it is essential to use mammals.
31 Suitable laboratory mammals for experimental ease of
32 manipulation include mice and rats. Larger yields may
33 be had from domestic farm animals such as cows, pigs,

1 goats and sheep. Intermediate between laboratory
2 animals and farm animals are such animals as rabbits,
3 which could be suitable producer animals for certain
4 proteins.

5
6 The 5' flanking sequence will generally include the
7 milk protein, e.g. beta-lactoglobulin (BLG),
8 transcription start site. For BLG it is preferred that
9 about 800 base pairs (for example 799 base pairs)
10 upstream of the BLG transcription start site be
11 included. In particularly preferred embodiments, at
12 least 4.2 kilobase pairs upstream be included.

13
14 The DNA coding for the protein other than BLG ("the
15 heterologous protein") may code for any desired protein
16 of interest. One particularly preferred category of
17 proteins of interest are plasma proteins. Important
18 plasma proteins include serine protease inhibitors,
19 which is to say members of the SERPIN family. An
20 example of such a protein is α_1 -antitrypsin. Other
21 serine protease inhibitors may also be coded for.
22 Other plasma proteins apart from serine protease
23 inhibitors include the blood factors, particularly
24 Factor VIII and Factor IX.

25
26 Proteins of interest also include proteins having a
27 degree of homology (for example at least 90%) with the
28 plasma proteins described above. Examples include
29 oxidation-resistant mutants and other analogues of
30 serine protease inhibitors such as AAT. These
31 analogues include novel protease inhibitors produced by
32 modification of the active site of α_1 -antitrypsin.
33 For example, if the Met-358 of AAT is modified to Val,

1 this replacement of an oxidation-sensitive residue at
2 the active centre with an inert valine renders the
3 molecule resistant to oxidative inactivation.
4 Alternatively, if the Met-358 residue is modified to
5 Arg, the molecule no longer inhibits elastase, but is
6 an efficient heparin-independent thrombin inhibitor
7 (that is to say, it now functions like anti-thrombin
8 III).

9
10 The protein-coding DNA has a partial complement of
11 natural introns or parts thereof. It is preferred in
12 some embodiments that all but one be present. For
13 example, the first intron may be missing but it is also
14 possible that other introns may be missing. In other
15 embodiments of the invention, more than one is missing,
16 but there must be at least one intron present in the
17 protein-coding DNA. In certain embodiments it is
18 preferred that only one intron be present.

19
20 Suitable 3'-sequences may be present. It may not be
21 essential for such sequences to be present, however,
22 particularly if the protein-coding DNA of interest
23 comprises its own polyadenylation signal sequence.
24 However, it may be necessary or convenient in some
25 embodiments of the invention to provide 3'-sequences
26 and 3'-sequences of BLG will be those of choice.
27 3'-sequences are not however limited to those derived
28 from the BLG gene.

29
30 Appropriate signal and/or secretory sequence(s) may be
31 present if necessary or desirable.

32

33

1 According to a second aspect of the invention, there is
2 provided a method for producing a substance comprising
3 a polypeptide, the method comprising introducing a DNA
4 construct as described above into the genome of an
5 animal in such a way that the protein-coding DNA is
6 expressed in a secretory gland of the animal.

7
8 The animal may be a mammal, expression may take place
9 in the mammary gland, for preference. The construct
10 may be inserted into a female mammal, or into a male
11 mammal from which female mammals carrying the construct
12 as a transgene can be bred.

13
14 Preferred aspects of the method are as described in
15 WO-A-8800239.

16
17 According to a third aspect of the invention, there is
18 provided a vector comprising a genetic construct as
19 described above. The vector may be a plasmid, phage,
20 cosmid or other vector type, for example derived from
21 yeast.

22
23 According to a fourth aspect of the invention, there is
24 provided a cell containing a vector as described above.
25 The cell may be prokaryotic or eukaryotic. If
26 prokaryotic, the cell may be bacterial, for example E.
27 coli. If eukaryotic, the cell may be a yeast cell or
28 an insect cell.

29
30 According to a fifth aspect of the invention, there is
31 provided a mammalian or other animal cell comprising a
32 construct as described above.

33

1 According to a sixth aspect of the invention, there is
2 provided a transgenic mammal or other animal comprising
3 a genetic construct as described above integrated into
4 its genome. It is particularly preferred that the
5 transgenic animal transmits the construct to its
6 progeny, thereby enabling the production of at least
7 one subsequent generation of producer animals.

8

9 The invention will now be illustrated by a number of
10 examples. The examples refer to the accompanying
11 drawings, in which:

12

13 FIGURES 1 to 10 show schematically one strategy used
14 for elaborating fusion genes comprising DNA sequence
15 elements from ovine beta-lactoglobulin and the gene(s)
16 of interest, in this case α_1 -antitrypsin, to be
17 expressed in the mammary gland of a mammal;

18

19 FIGURE 11 shows a Northern blot giving the results of
20 Example 2;

21

22 FIGURE 12 shows an RNase protection gel, referred to in
23 Example 2;

24

25 FIGURE 13 shows an Immuno blot of diluted milk samples
26 from transgenic and normal mice, referred to in Example
27 2;

28

29 FIGURE 14 shows a Western blot of milk whey samples
30 from normal and two transgenic sheep (Example 3);

31

32 FIGURE 15 shows Western blots of TCA-precipitated whey
33 samples from normal and transgenic mice (Example 3);

1 FIGURES 16a, 16b and 17 to 20 show schematically the
2 strategy used for elaborating a further strategy used
3 for elaborating fusion genes comprising DNA sequence
4 elements from ovine beta-lactoglobulin and the gene(s)
5 of interest, in this case Factor IX, to be expressed in
6 the mammary gland of a mammal.

7
8 EXAMPLE 1

9
10 General

11
12 Where not specifically detailed, recombinant DNA and
13 molecular biological procedures were after Maniatis et
14 al ("Molecular Cloning" Cold Spring Harbor (1982))
15 "Recombinant DNA" Methods in Enzymology Volume 68,
16 (edited by R. Wu), Academic Press (1979); "Recombinant
17 DNA part B" Methods in Enzymology Volume 100, (Wu,
18 Grossman and Moldgave, Eds), Academic Press (1983);
19 "Recombinant DNA part C" Methods in Enzymology Volume
20 101, (Wu, Grossman and Moldgave, Eds), Academic Press
21 (1983); and "Guide to Molecular Cloning Techniques",
22 Methods in Enzymology Volume 152 (edited by S.L. Berger
23 & A.R. Kimmel), Academic Press (1987). Unless
24 specifically stated, all chemicals were purchased from
25 BDH Chemicals Ltd, Poole, Dorset, England or the Sigma
26 Chemical Company, Poole, Dorset, England. Unless
27 specifically stated all DNA modifying enzymes and
28 restriction endonucleases were purchased from BCL,
29 Boehringer Mannheim House, Bell Lane, Lewes, East
30 Sussex BN7 1LG, UK.

31
32 [Abbreviations: bp = base pairs; kb = kilobase pairs,
33 AAT = alpha1-antitrypsin; BLG = beta-lactoglobulin;

1 FIX = factor IX; E. coli = Escherichia coli; dNTPs =
2 deoxyribonucleotide triphosphates; restriction
3 endonucleases are abbreviated thus e.g. BamHI; the
4 addition of -O after a site for a restriction
5 endonuclease e.g. PvuII-O indicates that the
6 recognition site has been destroyed]

7

8 A. PREPARATION OF CONSTRUCTIONS

9

10 Elaboration of Beta-Lactoglobulin Fusion Genes

11

12 The strategy used for elaborating fusion genes
13 comprising DNA sequence elements from the ovine
14 beta-lactoglobulin and the gene(s) of interest to be
15 expressed in the mammary gland is outlined in Figures 1
16 to 10. The approach utilises sequences derived from a
17 lambda clone, lambdaSS-1, which contains the gene for
18 ovine beta-lactoglobulin, and whose isolation and
19 characterisation is outlined in International Patent
20 Application No. WO-A-8800239 (Pharmaceutical Proteins
21 Ltd) and by Ali & Clark (1988) Journal of Molecular
22 Biology 199, 415-426.

23

24 The elaboration of seven constructs are described -
25 AATB, AATA, BLG-BLG, AATC, AATD, FIXD, and DELTA-A2 in
26 sections A1-A7 respectively. Construct AATB
27 constitutes the primary example and the other
28 constructs are included as comparative examples.

29

30 The nomenclature eg AATB is generally used to describe
31 the DNA construct without its associated bacterial
32 (plasmid) vector sequences. This form, lacking the
33 vector sequences, corresponds to that microinjected

1 into fertilised eggs and subsequently incorporated into
2 the chromosome(s) of the embryo.

3

4 A1 AATB - Construction of pIII-15BLGgAAT

5

6 The construct AATB is a hybrid gene which contains
7 sequence elements from the 5'-flanking region of the
8 ovine beta-lactoglobulin gene fused to sequences from
9 the human gene for alpha₁-antitrypsin. The features of
10 the AATB construct are summarised in Figure 6. The
11 sequences from the ovine beta-lactoglobulin gene are
12 contained in a SalI - SphI fragment of about 4.2kb
13 which contains (by inspection) a putative 'CCAAT box'
14 (AGCCAAGTG) [see Ali & Clark (1988) Journal of
15 Molecular Biology 199, 415-426]. In addition there are
16 ovine BLG sequences from this SphI to a PvuII site in
17 the 5'-untranslated region of the BLG transcription
18 unit. The sequence of this SphI - PvuII fragment is
19 shown in Figure 5. This latter fragment contains a
20 putative 'TATA box' (by inspection) [see Ali & Clark
21 (1988) Journal of Molecular Biology 199, 415-426]. The
22 mRNA cap site / transcription start point CACTCC as
23 determined by S1-mapping and RNase protection assays is
24 also contained within this fragment. Beyond the fusion
25 (PvuII-O) site are found sequences from a cDNA for
26 human alpha₁-antitrypsin and from the human
27 alpha₁-antitrypsin gene. The sequences from the 5'
28 fusion (TagI-O) site to the BamHI site 80 bp
29 downstream, include the initiation ATG methionine codon
30 for alpha₁-antitrypsin. The first nucleotide
31 (cytosine) in the AAT sequences (CGACAATG..., see
32 Figure 5) corresponds to the last nucleotide in exon I
33 of the AAT gene. The second nucleotide (guanosine) in

1 the AAT sequences (CGACAATG..., see Figure 5)
2 corresponds to the first nucleotide in exon II of the
3 AAT gene. The exclusion of intron I has been effected
4 by using DNA from a cDNA clone p8 α 1ppg (see below) as
5 the source of the first 80 bp of the AAT sequences in
6 AATB (TagI-0 to BamHI). The BamHI site corresponds to
7 that found in exon II of the AAT gene. Beyond this
8 BamHI site are approximately 6.5 kb of the human AAT
9 gene including - the rest of exon II, intron II, exon
10 III, intron III, exon IV, intron IV, exon V and about
11 1.5 kb of 3'-flanking sequences. Exon V contains the
12 AAT translation termination codon (TAA) and the
13 putative polyadenylation signal (ATTAAA). The signal
14 peptide for the peptide encoded by construct AATB is
15 encoded by the AAT cDNA sequence from ATGCCGTCT to
16 TCCCTGGCT (2 bp upstream from the BamHI site in exon
17 II.

18
19 Plasmid pSS1tgSE α 1AT

20 The subclone pSS1tgSE α 1AT was constructed as described
21 here and briefly in Example 2 of International Patent
22 Application No. WO-A-8800239 (Pharmaceutical Proteins
23 Ltd). This clone contains the cDNA sequences for human
24 α_1 -antitrypsin inserted into the 5'-untranslated
25 region of the ovine beta-lactoglobulin gene. The
26 plasmid p8 α 1ppg containing a full length cDNA encoding
27 an M variant of α_1 -antitrypsin was procured from
28 Professor Riccardo Cortese, European Molecular Biology
29 Laboratory, Meyerhofstrasse 1, D-6900 Heidelberg,
30 Federal Republic of Germany (Ciliberto, Dente & Cortese
31 (1985) Cell 41, 531-540). The strategy used in the
32 construct BLG-AAT or pSS1tgXSTARG, now known as AATA,
33 described in International Patent Application No.

1 WO-A-8800239 (Pharmaceutical Proteins Ltd) required
2 that the polyadenylation signal sequence at the 3' end
3 of the α_1 -antitrypsin cDNA be removed.

4
5 The polyadenylation signal was removed in the following
6 manner. Plasmid p8 α lppg DNA was digested with PstI and
7 the digestion products were separated by
8 electrophoresis in a preparative 1% agarose gel
9 containing 0.5 μ g/ml ethidium bromide (Sigma). The
10 relevant fragment of about 1400 bp was located by
11 illumination with a UV lamp (Ultra-Violet Products,
12 Inc, San Gabriel, California, USA). A piece of
13 dialysis membrane was inserted in front of the band and
14 the DNA fragment subsequently electrophoresed onto the
15 membrane. The DNA was eluted from the dialysis
16 membrane and isolated by use of an 'ElutipD' [Schleicher
17 and Schull, Postfach 4, D-3354, Dassel, W. Germany],
18 employing the procedure recommended by the
19 manufacturer. The gel purified 1400 bp PstI fragment
20 was digested with the TaqI, electrophoresed on a
21 preparative 1% agarose gel as described above. The
22 TaqI - PstI fragment of approximately 300 bp comprising
23 the 3' end of the α_1 -antitrypsin cDNA including the
24 polyadenylation signal sequence was eluted and purified
25 using an Elutip as described above, as was the TaqI -
26 TaqI fragment of 1093 bp containing the 5' portion of
27 the cDNA. The plasmid vector pUC8 (Pharmacia-LKB
28 Biotechnology, Pharmacia House, Midsummer Boulevard,
29 Central Milton Keynes, Bucks, MK9 3HP, UK) was digested
30 with AccI and PstI, phenol/chloroform extracted and DNA
31 recovered by ethanol precipitation. The 300 bp TaqI -
32 PstI fragment from p8 α lppg was ligated using T4 DNA
33 ligase to pUC8 cut with AccI and PstI and the ligation

1 products were used to transform E. coli strain DH-1
2 (Gibco-BRL, PO Box 35, Trident House, Renfrew Road,
3 Paisley PA3 4EF, Scotland, UK) to ampicillin
4 resistance. Plasmid DNA was isolated from ampicillin
5 resistant colonies. The correct recombinants were
6 identified by the release of a fragment of
7 approximately 300 bp on double digestion with AccI and
8 PstI. The plasmid generated was called pUC8.3'AT.3.

9
10 Plasmid pUC8.3'AT.3 was subjected to partial digestion
11 with BstNI and the fragment(s) corresponding to
12 linearised pUC8.3'AT.3 isolated from an agarose gel.
13 There are seven BstNI sites in pUC.3'AT.3, five in the
14 vector and two in the region corresponding to the
15 3'-untranslated sequences of alpha₁-antitrypsin. The
16 BstNI linearised and gel purified DNA was digested with
17 PstI which cuts in the pUC8 polylinker where it joins
18 the 3' end of the cDNA insert. The PstI digested DNA
19 was end repaired with T4 DNA polymerase in the presence
20 of excess dNTPs and self-ligated with T4 DNA ligase.
21 The BstNI - PstI fragment containing the
22 polyadenylation signal sequence is lost by this
23 procedure. The ligated material was used to transform
24 E. coli strain DH-1 to ampicillin resistance. Plasmid
25 DNA was isolated from ampicillin resistant colonies.
26 The correct clone was identified by restriction
27 analysis and comparison with pUC8.3'AT.3. The correct
28 clone was characterised by retention of single sites
29 for BamHI and HindIII, loss of a PstI site, and a
30 reduction in the size of the small PvuII fragment. The
31 correct clone was termed pB5.

32

33

1 Plasmid pB5 DNA was digested with AccI,
2 phenol/chloroform extracted and DNA recovered by
3 ethanol precipitation. AccI cleaved pB5 DNA was
4 treated with calf intestine alkaline phosphatase (BCL).
5 The reaction was stopped by adding EDTA to 10
6 millimolar and heating at 65°C for 10 minutes. The DNA
7 was recovered after two phenol/chloroform and one
8 chloroform extractions by precipitation with ethanol.
9 T4 DNA ligase was used to ligate the 1093 bp TaqI -
10 TaqI fragment described above to pB5, AccI cleaved and
11 phosphatased DNA and the ligation products were used to
12 transform E. coli strain HB101 (Gibco-BRL) to
13 ampicillin resistance. The identity of the correct
14 clone (pUC8 α 1AT.73) was verified by restriction
15 analysis - presence of a 909 bp HinfI fragment, a 1093
16 bp TaqI fragment, and a 87 bp BamHI fragment.

17

18 The α_1 -antitrypsin cDNA minus its polyadenylation
19 signal was excised from pUC8 α 1AT.73 as a 1300 bp AccI -
20 HindIII fragment and isolated from a preparative gel.
21 The 1300 bp AccI - HindIII fragment was end-repaired
22 with the Klenow fragment of E. coli DNA polymerase in
23 the presence of excess dNTPs. The fragment was ligated
24 into PvuII restricted, phosphatase treated pSSltgSE DNA
25 (see International Patent Application No. WO-A-8800239
26 (Pharmaceutical Proteins Ltd) to form pSSltgSE α 1AT
27 after transforming E. coli DH-1 to ampicillin
28 resistance.

29

30 Plasmid pIII-ISpB (see Figure 1)
31 pSSltgSE α 1AT DNA was linearised by digestion with SphI
32 which cuts at a unique site in the plasmid in a region
33 of DNA corresponding to the 5' flanking sequences of

1 the beta-lactoglobulin transcription unit. The DNA was
2 recovered after phenol/chloroform extractions by
3 precipitation with ethanol. The SphI linearised
4 plasmid was digested with BamHI which cuts at a unique
5 site in the plasmid in a region of DNA corresponding to
6 the mRNA sequences of alpha₁-antitrypsin. The 155 bp
7 SphI - BamHI fragment, comprising beta-lactoglobulin
8 sequences fused to alpha₁-antitrypsin sequences was
9 located in a 1% agarose gel and isolated by use of an
10 ElutipD as described above.

11

12 The plasmid pIII-ISpB was constructed by using T4 DNA
13 ligase to ligate the 155 bp SphI - BamHI fragment from
14 subclone pSS1tgSEα1AT into the plasmid vector
15 pPolyIII-I (Lathe, Vilotte & Clark, 1987, Gene 57,
16 193-201) which had been digested with SphI and BamHI.
17 [The vector pPolyIII-I is freely available from
18 Dr. A. J. Clark, AFRC Institute of Animal Physiology
19 and Genetics Research, West Mains Road, Edinburgh EH9
20 3JQ, UK.] Clones were isolated after transforming
21 competent E. coli DH5α cells (Gibco-BRL) to ampicillin
22 resistance. Plasmid DNA was prepared from the
23 ampicillin resistant colonies and screened for the
24 desired product. pIII-ISpB was confirmed as the
25 desired product by the retention of cleavage sites for
26 the enzymes BamHI and SphI and by the addition (when
27 compared to the vector pPolyIII-I) of a cleavage site
28 for the enzyme StuI. The StuI site is present in the
29 155 bp SphI - BamHI fragment isolated from
30 pSS-1tgSEα1AT.

31

32 Plasmid pIII-15BLGSpB (pAT2-3) (see Figure 2)
33 pIII-ISpB DNA was digested with the SphI and SalI.

1 SphI cuts at a unique site in the plasmid in a region
2 of DNA corresponding to the 5' flanking sequences of
3 the beta-lactoglobulin transcription unit. This site
4 represents the junction between the beta-lactoglobulin
5 sequences and the plasmid vector sequences. SalI cuts
6 at a unique site in the plasmid in the vector
7 polylinker sequences. The SphI/SalI digested pIII-ISpB
8 DNA was electrophoresed on a preparative 1% agarose gel
9 as described above. The SalI - SphI fragment of
10 approximately 2.2 kb was eluted and purified using an
11 Elutip as described above.

12
13 The plasmid DNA pSS-1tgXS (described in International
14 Patent Application No. WO-A-8800239 (Pharmaceutical
15 Proteins Ltd)) was digested with SphI and SalI and the
16 DNA electrophoresed on a 0.9% agarose gel. The
17 relevant SalI - SphI fragment, comprising approximately
18 4.2 kb of DNA sequences from the 5' flanking sequences
19 of the beta-lactoglobulin gene, was located by
20 illumination with ultra violet light and recovered by
21 use of an Elutip as described above.

22
23 The plasmid pIII-15BLGSpB was constructed by using T4
24 DNA ligase to ligate the 4.2 kb SalI - SphI fragment
25 described above into gel purified SalI - SphI digested
26 pIII-ISpB DNA. Clones were isolated after transforming
27 E. coli DH5 α (Gibco-BRL) to ampicillin resistance.
28 Plasmid DNA was prepared from the ampicillin resistant
29 colonies and screened for the desired product. The
30 correct product was verified by the presence of two
31 BamHI sites - one in the 4.2 kb fragment containing the
32 5' flanking sequences of beta-lactoglobulin and one in
33 the sequences corresponding to the α_1 -antitrypsin

1 mRNA. Cleavage of the correct product with BamHI
2 yields two fragments including one of approximately
3 1.75 kb which spans the cloning junctions (see
4 Figure 2).

5

6 Plasmid pIII-15BLGgAAT (AATB or G7) (see Figure 3)

7 An α_1 -antitrypsin DNA clone pATp7 was procured from
8 Dr. Gavin Kelsey, MRC Human Biochemical Genetics Unit,
9 The Galton Laboratory, University College London,
10 Wolfson House, 4 Stephenson Way, London NW1 2HE, UK.
11 This clone contains the entire α_1 -antitrypsin
12 transcription unit plus 348 bp of 5' and approximately
13 1500 bp of 3' flanking sequences as an insert of
14 approximately 12.3 kb in the BamHI site of a plasmid
15 vector pUC9 (Pharmacia-LKB Biotechnology, Pharmacia
16 House, Midsummer Boulevard, Central Milton Keynes,
17 Bucks, MK9 3HP, UK). The insert for clone pATp7 was
18 prepared by partial BamHI and partial BglIII digestion
19 of cosmid clone α ATc1 (Kelsey, Povey, Bygrave &
20 Lovell-Badge (1987) Genes and Development 1, 161-171).
21 The clone pATp7 contains the gene which encodes the M₁
22 allele, which is the most frequent at the Pi locus.
23 Most of the DNA sequence of this gene is reported by
24 Long, Chandra, Woo, Davie & Kurachi (1984) Biochemistry
25 23, 4828-4837.

26

27 Plasmid DNA from pATp7 was digested with BamHI and
28 electrophoresed in a 0.9% agarose gel. The relevant
29 BamHI fragment, comprising approximately 6500bp of
30 α_1 -antitrypsin sequences from the BamHI site in
31 exon II of this gene to a BamHI site in the 3' flanking
32 region was located and purified by use of an Elutip as
33 described above.

1 The plasmid pIII-15BLGSpB (also known as AT2-3) was
2 linearised by partial digestion with BamHI. There are
3 two BamHI sites in this plasmid one in the sequences
4 corresponding to the 5' flanking sequences of
5 beta-lactoglobulin and the other in the sequences
6 corresponding to the mRNA for alpha₁-antitrypsin. The
7 latter site is the desired site for insertion of the
8 6500 bp BamHI fragment from pATp7. The products of the
9 partial BamHI digestion of plasmid pIII-15BLGSpB were
10 electrophoresed in a 0.9% agarose gel. The fragment(s)
11 corresponding to linearised pIII-15BLGSpB were located
12 and purified using an Elutip as described above. It is
13 expected that this fragment preparation will contain
14 the two possible BamHI linearised molecules. BamHI
15 linearised, gel purified DNA was dissolved in TE (10 mM
16 Tris.HCl, 1 mM EDTA pH 8) and treated with calf
17 intestinal phosphatase (BCL) for 30 minutes at 37°C.
18 The reaction was stopped by adding EDTA to 10
19 millimolar and heating at 65°C for 10 minutes. The DNA
20 was recovered after two phenol/chloroform and one
21 chloroform extractions by precipitation with ethanol.

22
23 The plasmid pIII-15BLGgAAT was constructed by using T4
24 DNA ligase to ligate the 6500 bp BamHI fragment from
25 pATp7 into BamHI linearised, gel purified and
26 phosphatase treated pIII-15BLGSpB DNA. Clones were
27 isolated after transforming E. coli DH-5 (Gibco-BRL) to
28 ampicillin resistance. Plasmid DNA was purified from
29 the ampicillin resistant colonies and screened for the
30 desired product. The desired clones were characterised
31 by restriction analysis and, in particular, by the
32 presence of an SphI fragment of approximately 1.6 kb.
33 Plasmid DNA was prepared for one such clone (G7) and

1 given the nomenclature pIII-15BLGgAAT (also known as
2 AATB).

3
4 The diagnostic 1.6kb SphI fragment was subcloned from
5 pIII-15BLGgAAT into the SphI site of the M13 vector
6 M13tg130 (Kieny, Lathe & Lecocq (1983) Gene 26, 91-99).
7 The DNA sequence of 180 nucleotides from the SphI site
8 corresponding to that in the 5' flanking region of the
9 beta-lactoglobulin gene in a 3' direction through the
10 fusion point of the beta-lactoglobulin and
11 α_1 -antitrypsin sequences was determined by the
12 chain terminator reaction using a SequenaseTM kit (USB,
13 United States Biochemical Corporation, PO Box 22400,
14 Cleveland, Ohio 44122, USA) according to the
15 manufacturers instructions. The sequence of this
16 region is given in Figure 5.

17
18 Preparation of DNA for microinjection (see Figure 4)
19 The β -lactoglobulin/ α_1 -antitrypsin fusion gene insert
20 was excised from pIII-15BLGgAAT as follows. 25-50 μ g
21 aliquots of pIII-15BLGgAAT plasmid DNA were digested
22 with NotI and the digested material electrophoresed on
23 a 0.6% agarose gel. The larger fragment of
24 approximately 10.5 kb was visualised under ultra-violet
25 light and purified using an Elutip as described above.
26 Following ethanol precipitation of the DNA eluted from
27 the Elutip, the DNA was further purified as follows.
28 The DNA was extracted once with phenol/chloroform, once
29 with chloroform and was then precipitated with ethanol
30 twice. The DNA was washed with 70% ethanol, dried
31 under vacuum and dissolved in TE (10 mM Tris.HCl, 1mM
32 EDTA pH 8). All aqueous solutions used in these later
33 stages had been filtered through a 0.22 μ m filter.

1 Pipette tips were rinsed in filtered sterilised water
2 prior to use. The DNA concentration of the purified
3 insert was estimated by comparing aliquots with known
4 amounts of bacteriophage lambda DNA on ethidium bromide
5 stained agarose gels. The insert DNA was checked for
6 purity by restriction mapping.

7

8 **A2 AATA - Construction of pSS1tgXS α 1AT**

9

10 The construct AATA is analogous to the construct
11 BLG-FIX or pSS1tgXSFIX described in International
12 Patent Application No. WO-A-8800239 (Pharmaceutical
13 Proteins Ltd). The elaboration of AATA is outlined in
14 Example 2 of International Patent Application No.
15 WO-A-8800239 (Pharmaceutical Proteins Ltd) as a second
16 example of the generalised construct pSS1tgXSTARG. The
17 first stages of the construction of AATA (ie the
18 generation of the plasmid pSS1tgSE α 1AT) are described
19 above in section A1,

20

21 **A3 BLG-BLG - Construction of pSS1tgXSDELTA α 1aBLG** (see
22 Figures 7 and 8)

23

24 The construct is analogous to FIXA and AATA (generally
25 designated as pSS1tgXSTARG and specifically as BLG-FIX
26 and BLG-AAT in patent WO-A-8800239) ie, the cDNA for
27 ovine β -lactoglobulin has been inserted into the PvuII
28 site in the first exon of pSS1tgXSDELTA α 1a (see below).
29 pSS1tgXSDELTA α 1a is a variant of pSS1tgXS lacking the
30 ClaI restriction site found in exon 3 which should
31 cause a frameshift in the 2nd open reading frame in the
32 expected bicistronic message of BLG-BLG and premature
33 termination of any polypeptide being translated. It

1 was necessary to sabotage the 2nd open reading frame in
2 this manner in order that the polypeptides encoded by
3 the two open reading frames could be distinguished. In
4 order to generate this construct a full length BLG cDNA
5 had first to be made.

6

7 pUCBlacA

8 Two complimentary 44-mer oligonucleotides, synthesised
9 by the Oswell DNA Service, Department of Chemistry,
10 University of Edinburgh, and containing bases 117-159
11 of the ovine β -lactoglobulin cDNA sequence (Gaye *et al*,
12 (1986) *Biochimie* 68, 1097-1107) were annealed to
13 generate SalI and StyI complimentary termini. The
14 annealed oligonucleotides were then ligated using T4
15 DNA ligase to equimolar amounts of a gel purified 457
16 bp StyI - SmaI fragment from β -Lg 931 (Gaye *et al*, *op*
17 *cit*) and gel purified pUC19 (Pharmacia-LKB
18 Biotechnology, Pharmacia House, Midsummer Boulevard,
19 Central Milton Keynes, Bucks, MK9 3HP, UK) which had
20 been digested with SalI - SmaI. After transformation
21 of competent *E. coli* strain JM83 (see Messing (1979)
22 Recombinant DNA Technical Bulletin, NIH Publication No.
23 79-99, 2, No. 2 (1979), 43-48) the correct recombinant
24 was determined by restriction analysis.

25

26 pUCBlacB

27 pUCBlacA digested with SphI and StuI was ligated to
28 equimolar amounts of a gel purified 163 bp SphI - StuI
29 fragment from pSS1tgSE (described in patent
30 WO-A-8800239) using T4 DNA ligase. After
31 transformation of competent *E. coli* strain JM83 the
32 correct recombinant was determined by restriction
33 analysis.

1 pSS1tgXSDELTACla
2 After transformation of competent E. coli strain DL43
3 (relevant phenotype dam⁻, dcm⁻; also called GM119, gift
4 of Dr. D. Leach, Department of Molecular Biology,
5 University of Edinburgh, West Mains Road, Edinburgh
6 EH9, UK) with the plasmid pSS1tgXS plasmid DNA was
7 isolated and digested to completion with ClaI. The DNA
8 termini were end-repaired using the Klenow fragment of
9 E. coli DNA polymerase in the presence of excess dNTP's
10 prior to ligation with T4 DNA ligase in the presence of
11 1mM hexamine cobalt chloride, 25mM KCl ([to encourage
12 self-ligation (Rusche & Howard-Flanders (1985) Nucleic
13 Acids Research 13, 1997-2008)]). The ligation products
14 were used to transform competent DL43 and ClaI
15 deficient recombinants were confirmed by restriction
16 analysis.

17

18 pSS1tgSE_BLG
19 Equimolar amounts of gel purified pSS1tgSE, digested to
20 completion with PvuII and dephosphorylated with Calf
21 intestinal phosphatase (BCL), were ligated to a gel
22 purified 580 bp PvuII - SmaI fragment from pUC α lacB
23 using T4 DNA ligase. After transformation of competent
24 DH5 α (Gibco-BRL) the correct recombinant was confirmed
25 by restriction analysis.

26

27 pSE_BLG_3'
28 Equimolar amounts of gel purified pSS1tgSE_BLG digested
29 to completion with EcoRI were ligated to 3 (~4.3-5.3)
30 gel purified products of a partial EcoRI digestion of
31 pSS1tgXSDELTACla using T4 DNA ligase. After
32 transformation of competent DH5 α (Gibco-BRL) the
33 correct recombinant was identified by restriction
34 analysis.

1 pSS1tgXSDELTAclabLG

2 The gel purified ~3 kb SphI - HindIII fragment from
3 pSE_BLG_3' was ligated to equimolar amounts of gel
4 purified ~9.6 kb SphI-HindIII fragment from
5 pSS1tgDELTA^{Sph}XS (a derivative of pSS1tgXS lacking the
6 SphI restriction site in the polylinker region of the
7 vector pPoly1) using T4 DNA ligase. After
8 transformation of competent DL43 the construct was
9 confirmed by restriction analysis.

10

11 Isolation of DNA fragment for microinjection

12 pSS1tgXSDELTAclabLG was digested to completion with
13 BglIII and XbaI to pSS1tgXSDELTAclabLG was digested to
14 completion with BglIII and XbaI to liberate the insert
15 from the vector. The insert was recovered from an
16 agarose gel by electroelution onto dialysis membrane
17 (Smith (1980) Methods in Enzymology 65, 371-380).
18 After release from the membrane the DNA was
19 phenol/chloroform extracted, ethanol precipitated and
20 resuspended in 100 μ l H₂O ready for microinjection.

21

22 A4 AATC - Construction of pSS1pUCXSTGA.AAT (see
23 Figure 9)

24

25 This construct contains the cDNA sequences encoding
26 human alpha-1-antitrypsin (AAT) inserted into the
27 second exon of the ovine β -lactoglobulin (BLG) gene.
28 The aim was to determine whether or not inserting the
29 AAT cDNA sequences at a site distant from the BLG
30 promoter would improve the levels of expression. As
31 such, this construct comprises the intact first exon
32 and first intron of the BLG gene.

33

1 Since this construct contains two ATG codons (including
2 the normal BLG initiating methionine) in the first BLG
3 exon (ie before the sequences encoding AAT) an
4 'in-frame' termination codon (TGA) was introduced at
5 the junction point between BLG and AAT. This was
6 thought necessary to prevent the production of a fusion
7 protein between BLG and AAT. It will be noted that for
8 AAT protein to be produced from the expected
9 transcripts, reinitiation(at the natural initiating ATG
10 of AAT) of transcription will have to take place after
11 termination at this codon.

12

13 pSS1tgSE.TGA

14 Two oligonucleotides (5'CTTGTGATATCG3' and
15 5'AATTCGATATCAC3') were synthesised by the Oswell DNA
16 Service, Department of Chemistry, University of
17 Edinburgh. After annealing, the oligonucleotides
18 comprise a TGA stop codon, an EcoRV site and have
19 cohesive ends for a StyI and an EcoRI site,
20 respectively. The annealed oligonucleotides were
21 ligated to a gel purified StyI-EcoRI fragment of about
22 3.2 kb isolated from pSS1tgSE (pSS1tgSE is described in
23 International Patent Application No. WO-A-8800239
24 (Pharmaceutical Proteins ltd)). This will insert these
25 sequences at the StyI site which comprises nucleotides
26 20-25 of BLG-exon II and generates the plasmid
27 pSS1tgSE.TGA, in which the TGA stop codon is 'in frame'
28 with the sequences encoding BLG. Note the sequences 3'
29 to the BLG StyI site are replaced by the
30 oligonucleotides in this step. The ligation products
31 were used to transform E.coli strain DH5 α (Gibco-BRL)
32 to ampicillin resistance. The correct clone
33 (pSS1tgSE.TGA) was identified by restriction analysis -

1 retention of sites for EcoRI and SphI and acquisition
2 of a site for EcoRV.

3

4 pSS1tgSpX.TGA

5 pSS1tgSE.TGA was cleaved with EcoRI and the cohesive
6 termini were end-repaired by filling in with Klenow
7 fragment of E. coli DNA polymerase in the presence of
8 excess dNTPs. After end-repair the preparation was
9 cleaved with SphI and the insert fragment of about
10 800 bp (now SphI->EcoRI (blunt)) was isolated on a
11 preparative gel. Plasmid pBJ7 (this patent, see below,
12 section A4) was cleaved with SphI and PvuII and the
13 larger (about 4.3 kb) fragment isolated. Note that
14 this fragment contains the pPolyI vector sequences.
15 The SphI-EcoRI (blunt) fragment excised from
16 pSS1tgSE.TGA was ligated using T4 DNA ligase to the
17 SphI-PvuII fragment isolated from pBJ7 and the ligation
18 products used to transform E. coli strain DH5 α
19 (Gibco-BRL) to ampicillin resistance. The correct
20 recombinant plasmid pSS1tgSpX.TGA, which contains exon
21 I, intron I, part exon II, oligonucleotide, part exon 5
22 and exons 6 and 7 of the BLG gene, was identified by
23 restriction analysis.

24

25 pSS1pUCXS.TGA

26 The BLG 5' SaII - SphI fragment of about 4.2 kb was
27 isolated from pSS1tgXS (WO-A-8800239) and ligated to
28 equimolar amounts of the SphI-XbaI insert from
29 pSS1tgSpX.TGA and SaII-XbaI cleaved plasmid vector
30 pUC18 (Pharmacia-LKB Biotechnology, Pharmacia House,
31 Midsummer Boulevard, Central Milton Keynes, Bucks, MK9
32 3HP, UK). The ligation products were used to transform
33 E. coli strain DH5 α (Gibco-BRL) to ampicillin

1 resistance. The correct clone, pSS1pUCXS.TGA, was
2 identified by restriction analysis.

3
4 pSS1pUCXSAAT.TGA (AATC)
5 pSS1pUCXS.TGA contains a unique EcoRV site (derived
6 from the oligonucleotide) inserted in the second exon
7 which will cleave this plasmid 1 bp downstream of the
8 'in-frame' TGA. cDNA sequences can thus be inserted
9 into this plasmid downstream of the BLG sequences in
10 the second exon. This is exemplified by the
11 construction of pSS1pUCXSAAT.TGA (AATC) in which AccI -
12 HindIII fragment derived from pUC8 α 1AT.73 (this patent,
13 see Section A1 above) was inserted at the EcoRV site.
14 Plasmid pUC8 α 1AT.73 (described in section A1 above) was
15 digested with AccI and HindIII and the resulting
16 fragment containing the α_1 -antitrypsin cDNA minus
17 its polyadenylation signal was end-repaired using
18 Klenow fragment of E. coli DNA polymerase in the
19 presence of excess dNTPs. This blunt ended fragment
20 was gel purified and ligated using T4 DNA ligase to gel
21 purified pSS1pUCXS.TGA cleaved with EcoRV and
22 dephosphorylated to prevent recircularisation. After
23 transformation of competent E. coli strain DH5 α
24 (Gibco-BRL) with the ligation products, the correct
25 clone was identified by restriction enzyme analysis.

26
27 A5 Construction of AATD (pBJ16) (see Figure 10)
28 This construct contains the cDNA for human
29 α_1 -antitrypsin flanked by BLG sequences. The 5'
30 flanking sequences include the SalI to PvuII-0 BLG
31 sequences also present in AATA and AATB. The fusion
32 point between the BLG and AAT sequences is in the
33 5'-untranslated region of the BLG first exon as is the

1 case in AATA, FIXA and AATB. The 3' flanking sequences
2 comprise exons 6 and 7 of BLG and the 3' flanking
3 sequences of the BLG gene as far as the XbaI site.
4 This construct contains no introns and was designed to
5 examine whether the 5' and 3' BLG sequences described
6 above are sufficient to direct efficient mammary
7 specific expression of cDNAs encoding human plasma
8 proteins as exemplified by that for AAT.

9

10 Plasmid pSSltgSpX

11 The gel purified SphI - XbaI restriction fragment of
12 about 6.6 kb from pSSltgXS (described in patent
13 WO-A-8800239) was ligated using T4 DNA ligase to gel
14 purified pPolyI (Lathe, Vilotte & Clark, 1987, Gene 57,
15 193-201) (also described in patent WO-A-8800239)
16 digested with SphI and XbaI. [The vector pPolyI is
17 freely available from Professor R. Lathe, LGME-CNRS and
18 U184 INSERM, 11 rue Humann, 67085, Strasbourg, France.]
19 After transformation of competent E. coli strain DHR α
20 (Gibco-BRL) the correct clone was identified by
21 restriction enzyme analysis.

22

23 Plasmid pBJ5

24 The gel purified PvuII restriction fragment containing
25 the origin of replication from pSSltgSpX was
26 self-ligated using T4 DNA ligase in the presence of 1mM
27 hexamine cobalt chloride, 25mM KCl [to encourage
28 self-ligation (Rusche & Howard-Flanders (1985) Nucleic
29 Acids Research 13, 1997-2008)]. After transformation
30 of competent E. coli strain DHR α (Gibco-BRL) the
31 correct clone was identified by restriction enzyme
32 analysis.

33

1 Plasmid pUCBlacA

2 See example 1 A3 for a description of pUCBlacA

3

4 Plasmid pBJ7

5 The gel purified HincII - SmaI restriction fragment
6 from pUCBlacA was ligated using T4 DNA ligase to gel
7 purified pBJ5 linearised by partial digestion with
8 SmaI. After transformation of competent E. coli strain
9 DH5 α (Gibco-BRL) the correct clone was identified by
10 restriction enzyme analysis.

11

12 Plasmid pBJ8

13 The gel purified PvuII restriction fragment containing
14 the origin of replication from pBJ7 was self-ligated
15 using T4 DNA ligase in the presence of 1mM hexamine
16 cobalt chloride, 25mM KCl (to encourage self-ligation
17 [Rusche & Howard-Flanders (1985) Nucleic Acids Research
18 13, 1997-2008]). After transformation into competent
19 E. coli strain DH5 α (Gibco-BRL) the correct clone was
20 identified by restriction enzyme analysis.

21

22 Plasmid pBJ12

23 Plasmid pUC8 α 1AT.73 (described in section A1 above) was
24 digested with AccI and HindIII and the resulting
25 fragment containing the α_1 -antitrypsin cDNA minus
26 its polyadenylation signal was end-repaired using
27 Klenow fragment of E. coli DNA polymerase in the
28 presence of excess dNTPs. This blunt ended fragment
29 was gel purified and ligated using T4 DNA ligase to gel
30 purified pBJ8 linearised with PvuII. After
31 transformation of competent E. coli strain DH5 α
32 (Gibco-BRL) the correct clone was identified by
33 restriction enzyme analysis.

1 Plasmid pBJ1
2 Plasmid pSSltgSpS (described in this patent, see A7
3 below) was digested with BglIII and end-repaired using
4 the Klenow fragment of E. coli DNA polymerase in the
5 presence of excess dNTPs. The blunt-ends were modified
6 using HindIII synthetic linkers (New England Biolabs
7 Inc, 32 Tozer Road, Beverly, MA 01915-5510, USA) and
8 the resulting fragment self-ligated using T4 DNA ligase
9 in the presence of 1mM hexamine cobalt chloride, 25mM
10 KCl (to encourage self-ligation [Rusche &
11 Howard-Flanders (1985) Nucleic Acids Research 13,
12 1997-2008]). After transformation of competent E. coli
13 strain DH5 α (Gibco-BRL) the correct clone was
14 identified by restriction enzyme analysis.

15
16 Plasmid pBJ16 (AATD)
17 The gel purified HindIII - SphI fragment from pBJ1 and
18 the gel purified SphI - XbaI fragment from pBJ12 were
19 ligated using T4 DNA ligase to gel purified pUC19
20 (Pharmacia-LKB Biotechnology, Pharmacia House,
21 Midsummer Boulevard, Central Milton Keynes, Bucks, MK9
22 3HP, UK) digested with HindIII and XbaI. After
23 transformation of competent E. coli strain DH5 α
24 (Gibco-BRL) the correct clone was identified by
25 restriction enzyme analysis.

26
27 Isolation of AAT-D fragment from pBJ16 for
28 microinjection
29 Plasmid pBJ16 was digested with HindIII and XbaI and
30 the resulting 8.0 kb AATD fragment was isolated from a
31 gel using DE81 paper (Dretzen et al (1981) Analytical
32 Biochemistry 112, 285-298). After separation from the
33 DE81 paper the DNA was phenol/chloroform extracted,

1 ethanol precipitated and finally resuspended in TE
2 buffer (10 mM Tris-HCl, 1mM EDTA pH 8) ready for
3 microinjection.

4
5 **A6 FIXD - Construction of pBJ17**

6
7 The procedure of Example 1 A5 (construction of AATD) is
8 repeated, except that the DNA sequence encoding the
9 polypeptide of interest encodes Factor IX. A NheI -
10 HindIII fragment comprising 1553 bp of the insert from
11 p5'G3'CVI [see International Patent Application No.
12 WO-A-8800239 (Pharmaceutical Proteins Ltd)] was
13 inserted into the PvuII site of pBJ8 as described above
14 for pBJ12.

15
16 **A7 DELTA-A2 - Construction of pSSltgXDELTA-AvaII**
17 **(DELTA A2)**

18
19 This construct contains the minimum ovine
20 beta-lactoglobulin sequences that have so far been
21 shown in transgenic mice to result in tissue-specific
22 expression of the protein during lactation. The
23 complete sequence of this construct can be found in
24 Harris, Ali, Anderson, Archibald & Clark (1988),
25 Nucleic Acids Research 16 (in press).

26
27 **Plasmid pSSltgSpS**

28 The gel purified SalI - SphI restriction fragment of
29 approximately 4.2 kb isolated from pSSltgXS (described
30 in patent WO-A-8800239) was ligated, using T4 DNA
31 ligase, with equimolar amounts of gel purified pPolyI
32 (Lathe, Vilotte & Clark, 1987, Gene 57, 193-201)
33 digested with SalI and SphI. [The vector pPolyI is

1 freely available from Professor R. Lathe, LGME-CNRS and
2 U184 INSERM, 11 rue Humann, 67085 Strasbourg, France.]
3 After transformation of competent E. coli strain DH1
4 (Gibco-BRL) the correct clone was identified by
5 restriction analysis.

6

7 Plasmid pSS1tgSpDELTA-AvaII

8 Plasmid pSS1tgSpS was partially digested with AvaI
9 followed by digestion to completion with SalI. The
10 ends of the resultant DNA fragments were end-repaired
11 using the Klenow fragment of E. coli DNA polymerase in
12 the presence of excess dNTPs. After ligation using T4
13 DNA ligase in the presence of 1mM hexamine cobalt
14 chloride, 25mM KCl [to encourage self-ligation (Rusche
15 & Howard-Flanders (1985) Nucleic Acids Research 13,
16 1997-2008)], the DNA was used to transform competent
17 DH1 (Gibco-BRL). The correct AvaI deletion recombinant
18 was confirmed by restriction analysis.

19

20 Plasmid pSS1tgXDELTA-AvaII

21 The gel purified ~800 bp SphI - BqIII fragment from
22 pSS1tgSpDELTA-AvaII; ~6.5 kb SphI - XbaI fragment from
23 pSS1tgXS; and pPolyI digested with BqIII - XbaI were
24 ligated in approximately equimolar ratios using T4 DNA
25 ligase then used to transform competent DH1
26 (Gibco-BRL). The identity of the correct recombinant
27 was confirmed by restriction analysis.

28

29 Isolation of DNA fragment for injection

30 pSS1tgXDELTA-AvaII was digested to completion with
31 BqIII and XbaI to release the ~7.4 kb insert from the
32 vector. The insert was recovered from an agarose gel
33 using DE81 paper (Dretzen et al (1981) Analytical

1 Biochemistry 112, 295-298). After separation from the
2 DE81 paper the DNA was phenol/chloroform extracted,
3 ethanol precipitated and resuspended in 100 μ l TE ready
4 for microinjection. Alternatively, the insert was
5 recovered from an agarose gel by electroelution onto
6 dialysis membrane (Smith (1980) Methods in Enzymology
7 65, 371-380). After release from the membrane the DNA
8 was phenol/chloroform extracted, ethanol precipitated
9 and resuspended in 100 μ l H₂O ready for microinjection.

10

11 B. CONSTRUCTION OF TRANSGENIC ANIMALS

12

13 MICE

14

15 Procedures are similar to those described by Hogan,
16 Costantini and Lacy in "Manipulating the Mouse Embryo:
17 A Laboratory Manual" Cold Spring Harbor Laboratory
18 (1986).

19

20 Collection of fertilised eggs

21

22 Mice used for the collection of fertilised eggs are F₁
23 hybrids between the C57BL/6 and CBA inbred strains of
24 mice. C57BL/6 females and CBA males are obtained from
25 Harlan Olac Ltd (Shaw's Farm, Bicester OX6 OTP,
26 England) and used for the breeding of F₁ hybrids. The
27 mice are housed in controlled light conditions (lights
28 on at 03.00h, lights off at 17.00h). To induce
29 superovulation, adult female mice are injected with 5
30 international units of Pregnant Mares Serum
31 Gonadotropin (Cat. No. 4877, Sigma Chemical Company,
32 Poole, Dorset, England) in 0.1 ml of distilled water,
33 at 15.00h followed 46 to 48 hours later by injection of

1 5 international units of Human Chorionic Gonadotropin
2 (HCG) (Cat. No. CG-10, Sigma Chemical Company, Poole,
3 Dorset, England) in 0.1 ml of distilled water.
4 Following HCG injection, the females are housed
5 individually with mature C57BL/6 X CBA F₁ male mice for
6 mating. The following morning, mated female mice are
7 identified by the presence of a vaginal plug.

8
9 Mated females are killed by cervical dislocation. All
10 subsequent procedures are performed taking precautions
11 to avoid bacterial and fungal contamination. Oviducts
12 are excised and placed in M2 culture medium (Hogan,
13 Costantini and Lacy "Manipulating the Mouse Embryo: A
14 Laboratory Manual" Cold Spring Harbor Laboratory (1986)
15 pp254-256). The fertilised eggs are dissected out of
16 the ampullae of the oviducts into M2 containing
17 300 µg/ml hyaluronidase (Type IV-S, Cat. No. H3884,
18 Sigma Chemical Company, Poole, Dorset, England) to
19 release the cumulus cells surrounding the fertilised
20 eggs. Once the eggs are free of cumulus, they are
21 washed free of hyaluronidase and, until required for
22 injection, are kept at 37°C either in M2 in a
23 humidified incubator, or in a drop (100 - 200 µl) of
24 Medium No. 16 (Hogan, Costantini and Lacy "Manipulating
25 the Mouse Embryo: A Laboratory Manual" Cold Spring
26 Harbor Laboratory (1986) pp254-255, and 257), under
27 mineral oil (Cat. No. 400-5, Sigma Chemical Company,
28 Poole, Dorset, England) in an atmosphere of 95% air, 5%
29 CO₂.

30

31 Injection of DNA

32

33 The DNA to be injected is diluted to approximately

1 1.5 $\mu\text{g}/\text{ml}$ in AnalaR water (Cat. No. 10292 3C, BDH
2 Chemicals, Burnfield Avenue, Glasgow G46 7TP,
3 Scotland), previously sterilised by filtration through
4 a 0.2 μm pore size filter (Cat. No. SM 16534,
5 Sartorius, 18 Avenue Road, Belmont, Surrey SM2 6JD,
6 England). All micropipette tips and microcentrifuge
7 tubes used to handle the DNA and diluent are rinsed in
8 0.2 μm -filtered water, to remove particulate matter
9 which could potentially block the injection pipette.
10 The diluted DNA is centrifuged at 12000 x g for at
11 least 15 minutes to allow any particulate matter to
12 sediment or float; a 20 μl aliquot is removed from just
13 below the surface and used to fill the injection
14 pipettes.

15
16 Injection pipettes are prepared on the same day they
17 are to be used, from 15cm long, 1.0mm outside diameter,
18 thin wall, borosilicate glass capillaries, with
19 filament (Cat. No. GC100TF-15; Clark Electromedical
20 Instruments, PO Box 8, Pangbourne, Reading, RG8 7HU,
21 England), by using a microelectrode puller (Campden
22 Instruments, 186 Campden Hill Road, London, England).
23 DNA (approximately 1 μl) is introduced into the
24 injection pipettes at the broad end; it is carried to
25 the tip by capillary action along the filament. To
26 prevent evaporation of water from the DNA solution,
27 approximately 20 μl Fluorinert FC77 (Cat. No. F4758,
28 Sigma Chemical Company, Poole, Dorset, England) is laid
29 over the DNA solution. The filled injection pipettes
30 are stored at 4°C until required.

31
32 The holding pipette (used to immobilise the eggs for
33 microinjection) is prepared from 10cm long, 1.0mm

1 outside diameter, borosilicate glass capillaries (Cat.
2 No. GC100-10; Clark Electromedical Instruments, PO Box
3 8, Pangbourne, Reading RG8 7HU, England). The glass is
4 heated over a small flame and pulled by hand to give a
5 2 - 4 cm long section with a diameter of 80 - 120 μ m.
6 Bends are introduced into the pipette, the glass is
7 broken and the tip is polished using a microforge
8 (Research Instruments, Kernick Road, Penryn TR10 9DQ,
9 England).

10

11 A cover slip chamber is constructed in which to
12 micromanipulate the eggs. The base of the cover-slip
13 chamber is a 26 x 76 x (1 - 1.2)mm microscope slide
14 (Cat. No. ML330-12, A and J Beveridge Ltd, 5 Bonnington
15 Road Lane, Edinburgh EH6 5BP, Scotland) siliconised
16 with 2% dimethyldichlorosilane (Cat. No. 33164 4V, BDH
17 Chemicals, Burnfield Avenue, Glasgow G46 7TP, Scotland)
18 according to the manufacturer's instructions; two glass
19 supports (25 x 3 x 1 mm, cut from microscope slides)
20 are fixed onto the slide with high vacuum silicone
21 grease (Cat. No. 33135 3N, BDH Chemicals, Burnfield
22 Avenue, Glasgow G46 7TP, Scotland) parallel to and
23 approximately 2mm from the long sides of the slide,
24 half way along the length of the slide. A further two
25 glass supports are fixed on top of the first pair, and
26 the top surface is smeared with silicone grease.
27 300 μ l of medium M2 are pipetted into the space between
28 the supports, and a 22 x 22 mm cover-slip (Cat. No.
29 ML544-20, A and J Beveridge Ltd, 5 Bonnington Road
30 Lane, Edinburgh EH6 5BP, Scotland) is lowered onto the
31 supports, a seal being formed by the grease.
32 Dow-Corning fluid (50 cs) (Cat. No. 63006 4V, BDH
33 Chemicals, Burnfield Avenue, Glasgow G46 7TP, Scotland)

1 is pipetted into the open ends of the chamber, to cover
2 the medium.

3

4 Batches of eggs (30 to 100) are placed into a
5 cover-slip chamber for manipulation. The chamber is
6 mounted on the microscope (Diaphot, Nikon (UK) Ltd,
7 Haybrooke, Telford, Shropshire, England) which has 4x
8 bright field, 10x phase contrast and 40x differential
9 interference contrast (DIC) objectives, and 10x
10 eyepieces. Mechanical micromanipulators (Cat. Nos.
11 520 137 and 520 138, E. Leitz (Instruments) Ltd, 48
12 Park Street, Luton, England) are mounted adjacent to
13 the microscope and are used to control the positions of
14 the holding and injection pipettes.

15

16 The holding pipette and DNA-containing injection
17 pipette are mounted in modified instrument tubes (Cat.
18 No. 520 145, E. Leitz (Instruments) Ltd, 48 Park
19 Street, Luton, England) which are in turn mounted onto
20 the micromanipulators via single unit (Cat. No.
21 520 142, E. Leitz (Instruments) Ltd, 48 Park Street,
22 Luton, England) and double unit (Cat. No. 520 143, E.
23 Leitz (Instruments) Ltd, 48 Park Street, Luton,
24 England) instrument holders, respectively. The
25 instrument tubes are modified by gluing onto Clay Adams
26 "Intramedic" adapters (2.0-3.5 mm tubing to female
27 Luer, Cat. No. 7543D, Arnold R. Horwell Ltd, 2
28 Grangeway, Kilburn High Road, London NW6 2BP, England),
29 which are used to connect the instrument tubes to
30 approximately 2 metres of polythene tubing (1.57 mm
31 inside diameter, 2.9 mm outside diameter, Cat. No.
32 F21852-0062, R.B. Radley & Co, Ltd, London Road,
33 Sawbridgeworth, Herts CM21 9JH, England), further

1 "Intramedic" adapters are connected to the other ends
2 of the polythene tubing to facilitate connection to the
3 syringes used to control the holding and injection
4 pipettes.

5
6 Injection is controlled using a 20ml or a 100ml glass
7 syringe (Cat. Nos. M611/20 and M611/31, Fisons, Bishop
8 Meadow Road, Loughborough LE11 0RG, England), the
9 plunger of which is lightly greased with high vacuum
10 silicone grease (Cat. No. 33135 3N, BDH Chemicals,
11 Burnfield Avenue, Glasgow G46 7TP, Scotland).

12
13 Holding of eggs is controlled with an Agla micrometer
14 syringe (Cat. No. MS01, Wellcome Diagnostics, Temple
15 Hill, Dartford DA1 5AH, England), which is fitted with
16 a light spring around the plunger. The Agla syringe is
17 connected via a 3-way stopcock (Cat. No. SYA-580-L),
18 Gallenkamp, Belton Road West, Loughborough LE11 0TR,
19 England), to the "Intramedic" adapter, the third port
20 of the stopcock is connected to a reservoir of
21 Fluorinert FC77 (Cat. No. F 4758, Sigma Chemical
22 Company, Poole, Dorset, England), which fills the Agla
23 syringe, polythene tubing, instrument tube and holding
24 pipette.

25
26 The tip of the injection pipette is broken off against
27 the holding pipette, to increase the tip diameter to a
28 size which allows free passage of the DNA solution and
29 which is small enough to allow injection without lethal
30 damage to the eggs ($\leq 1 \mu\text{m}$). The flow of DNA through
31 the pipette tip is checked by viewing under phase
32 contrast conditions whilst pressure is applied to the
33 injection syringe (the DNA solution will appear as a
34 bright plume emerging from the tip of the pipette).

1 One by one, fertilised eggs are picked up on the
2 holding pipette, and one or both pronuclei brought into
3 the same focus as the injection pipette (using the 40x
4 objective and DIC conditions; the correction ring on
5 the objective is adjusted for optimum resolution). The
6 injection pipette is inserted into one of the
7 pronuclei, avoiding the nucleoli, pressure is applied
8 to the injection syringe and once swelling of the
9 pronucleus is observed, pressure is released and the
10 injection pipette is immediately withdrawn. When
11 pipettes block, the blockage may be cleared by
12 application of high pressure on the injection syringe
13 or by breaking off a further portion of the tip. If
14 the blockage cannot be cleared, or if the pipette tip
15 becomes dirty, the pipette is replaced.

16
17 After injection, the eggs are cultured overnight in
18 medium No. 16 under oil in an atmosphere of 5% CO₂.
19 Eggs which cleave to two cells during overnight culture
20 are implanted into pseudopregnant foster mothers.

21
22 Random-bred albino (MF1, Harlan Olac Ltd, Shaw's Farm,
23 Bicester, OX6 OTP, England) female mice are mated with
24 vasectomised (Hogan, Costantini and Lacy, "Manipulating
25 the Mouse Embryo: A Laboratory Manual" Cold Spring
26 Harbor Laboratory (1986); Rafferty, "Methods in
27 experimental embryology of the mouse", The Johns
28 Hopkins Press, Baltimore, USA (1970)) MF1 male mice.
29 The matings are performed one day later than those of
30 the superovulated egg donors. MF1 females which have a
31 detectable vaginal plug the following morning are used
32 as foster mothers. The ideal weight of foster mothers
33 is 25 to 30g. Each foster mother is anaesthetised by

1 intraperitoneal injection of Hypnorm/Hypnovel (10 μ l/g
2 body weight) at 2/3 the concentration recommended by
3 Flecknell (Veterinary Record, 113, 574) (Hypnorm: Crown
4 Chemical Co, Ltd, Lamberhurst, Kent TN3 8DJ, England;
5 Hypnovel: Roche Products Ltd, PO Box 8, Welwyn Garden
6 City, Herts AL7 3AY, England) and 20 to 30 2-cell eggs
7 are transferred into one oviduct by the method
8 described by Hogan, Costantini and Lacy ("Manipulating
9 the Mouse Embryo: A Laboratory Manual" Cold Spring
10 Harbor Laboratory (1986)). As an option, to minimise
11 bleeding from the ovarian bursa, 2 μ l of 0.01% (w:v)
12 epinephrine bitartrate (Cat. No. E4375, Sigma Chemical
13 Company, Poole, Dorset, England) dissolved in distilled
14 water is applied to the bursa a few minutes before
15 tearing it. Foster mothers are allowed to deliver
16 their offspring naturally unless they have not done so
17 by 19 days after egg transfer, in which case the pups
18 are delivered by hysterectomy, and are fostered.
19 Following normal mouse husbandry, the pups are weaned
20 at 3 to 4 weeks of age and housed with other mice of
21 the same sex only.

22

23 Transgenic female mice may be used for the breeding of
24 subsequent generations of transgenic mice by standard
25 procedures and/or for the collection of milk and RNA.
26 Transgenic male mice are used to breed subsequent
27 generations of transgenic mice by standard procedures.
28 Transgenic mice of subsequent generations are
29 identified by analysis of DNA prepared from tails, as
30 described below.

31

32

33

1 SHEEP

2

3 The generation of transgenic sheep is described in
4 detail in International Patent Application No.
5 WO-A-8800239 (Pharmaceutical Proteins Ltd) and by
6 Simons, Wilmut, Clark, Archibald, Bishop & Lathe (1988)
7 Biotechnology 6, 179-183.

8

9 C. IDENTIFICATION OF TRANSGENIC INDIVIDUALS

10

11 MICE

12

13 When the pups are at least 4 weeks of age, a biopsy of
14 tail is taken for the preparation of DNA. The pups are
15 anaesthetised by intraperitoneal injection of
16 Hypnorm/Hypnovel (10 µl/g body weight) at 1/2 the
17 concentration recommended by Flecknell (Veterinary
18 Record, 113, 574). Once anaesthetised, a portion of
19 tail (1 to 2 cm) is removed by cutting with a scalpel
20 which has been heated in a Bunsen flame; the hot blade
21 cauterises the wound and prevents bleeding.

22

23 The tail segments are digested with proteinase
24 K 200 µg/ml (Sigma) in tail buffer [0.3 M NaAcetate
25 (not titrated), 10 mM Tris-HCl pH 7.9, 1 mM EDTA pH
26 8.0, 1% SDS] overnight with shaking at 37°C. The
27 following day the digests are vortexed briefly to
28 disaggregate the debris. Aliquots of digested tail are
29 phenol/chloroform extracted once, chloroform extracted
30 once and then DNA is recovered by precipitation with an
31 equal volume of isopropanol.

32

33

1 'Tail DNA' is digested with restriction enzyme(s), and
2 subjected to agarose gel electrophoresis. The
3 separated DNA is then 'Southern' blotted to HybondTM N
4 (Amersham) nylon membranes as described in the Amersham
5 Handbook 'Membrane transfer and detection methods'
6 (P1/162/86/8 published by Amersham International plc,
7 PO Box 16, Amersham, Buckinghamshire HP7 9LL, UK). DNA
8 bound to the membranes is probed by hybridisation to
9 appropriate ³²P labelled DNA sequences (eg the
10 construct DNAs). The DNA probes are labelled with ³²P
11 by nick-translation as described in 'Molecular Cloning:
12 a Laboratory Manual' (1982) by Maniatis, Fritsch and
13 Sambrook, published by Cold Spring Harbor Laboratory,
14 Box 100, Cold Spring Harbor, USA. Alternatively DNA
15 probes are labelled using random primers by the method
16 described by Feinberg and Vogelstein (1984) Analytical
17 Biochemistry 137, 266-267. Briefly: The plasmid or
18 phage is cleaved with the appropriate restriction
19 enzymes and the desired fragment isolated from an
20 agarose gel. The labelling reaction is carried out at
21 room temperature by adding the following reagents in
22 order: H₂O, 6 µl OLB*, 1.2 µl BSA, DNA (max. 25 ng),
23 4 µl ³²P labelled dCTP (PB10205, Amersham plc, Amersham
24 UK), 1 µl (1 unit) Klenow Polymerase (BCL) to a final
25 volume of 30 µl.

26

27 *OLB comprises solution A: 625 µl 2M Tris, pH 8.0 + 25
28 µl 5M MgCl₂ + 350 µl H₂O + 18 µl 2-mercaptoethanol
29 (Sigma); solution B, 2M HEPES (Sigma), titrated to pH
30 6.6 with NaOH; solution C, Hexa deoxyribonucleotides
31 (Pharmacia-LKB Biotechnology Cat. No. 27-2166-01). The
32 labelling reaction is allowed to run overnight and then
33 the reaction stopped by the addition of 70 µl stop

1 solution (20 mM NaCl, 20 mM Tris pH 7.5, 2mM EDTA,
2 0.25% SDS, 1 μ M dCTP). Incorporation is assessed by
3 TCA precipitation and counting Cerenkov emission.

4
5 Hybridisations are carried out in sealed plastic bags
6 by a modification of the procedure described by Church
7 and Gilbert (1984). Proceedings of the National
8 Academy of Sciences (USA) 81, 1991-1995. Briefly: the
9 probe is used at a concentration of 1.5×10^6 Cerenkov
10 counts/ml of hybridisation buffer (HB: 0.5M sodium
11 phosphate pH 7.2, 7% SDS, 1mM EDTA). Firstly, the
12 membrane is prehybridised for 5 minutes in HB (15ml of
13 buffer per 20 cm² membrane) in the plastic bag at 65°C.
14 The probe is denatured by boiling and added to the same
15 volume of fresh HB. The plastic bag is cut open and
16 the prehybridisation solution drained and then the HB +
17 probe added and the bag re-sealed. The bag and
18 contents are incubated overnight on a rotary shaker at
19 65°C. After hybridisation the membrane is washed in 40
20 mM sodium phosphate, 1% SDS and 1mM EDTA three times
21 for ten minutes at 65°C and then a final wash is
22 carried out for 15-30 minutes at this temperature.
23 Washing is monitored with a hand-held Geiger counter.
24 The stringency of the washings may be adjusted
25 according to the particular needs of the experiment.
26 After the last wash the membrane is blotted dry and
27 then placed on a dry piece of Whatman filter paper and
28 wrapped in Saran-wrap. The membrane is exposed to
29 X-ray film (Agfa CURIX RP-1) using an X-ray cassette at
30 - 70°C for one or more days.

31
32 By comparison with known amounts of construct DNA
33 treated in the same manner DNA from transgenic

1 individuals can be identified and the number of copies
2 of the construct DNA which have been integrated into
3 the genome can be estimated.

4

5 The same methods are used to identify transgenic
6 offspring of the founder transgenic individuals.

7

8 SHEEP

9

10 The identification of transgenic sheep is described in
11 detail in International Patent Application No.
12 WO-A-8800239 (Pharmaceutical Proteins Ltd).

13

14 D. ANALYSIS OF EXPRESSION - METHODS

15

16 Collection of Mouse Milk

17

18 Female mice (at least 7 weeks of age) are housed
19 individually with adult male mice for mating. After 17
20 days, the male mice are removed from the cage and the
21 female mice are observed daily for the birth of
22 offspring. Milk and/or RNA are collected 11 days after
23 parturition.

24

25 For the collection of milk, the pups are separated from
26 the lactating female mice to allow the build-up of milk
27 in the mammary glands. After at least 3 hours, 0.3
28 international units of oxytocin (Sigma, Cat. No.
29 O 4250) in 0.1 ml of distilled water are administered
30 by intraperitoneal injection, followed after 10 minutes
31 by intraperitoneal injection of Hypnorm/Hypnovel
32 anaesthetic (10 μ l/g body weight) at 2/3 the
33 concentration recommended by Flecknell (Veterinary

1 Record, 113, 574). When fully anaesthetised, the
2 mammary glands are massaged to expel milk, which is
3 collected in 50 μ l capillary tubes (Drummond Microcaps,
4 Cat. No. PP600-78, A and J Beveridge Ltd, 5 Bonnington
5 Road Lane, Edinburgh EH6 5BP, Scotland).
6
7 Mouse milk is diluted 1:5 in distilled water and
8 centrifuged in an Eppendorf 5415 centrifuge (BDH) to
9 remove fat. To make whey, 1.0 M HCl was added to give
10 a final pH of 4.5, thus precipitating the caseins which
11 were then removed by centrifugation in an Eppendorf
12 5415 centrifuge. Diluted milk or whey samples were
13 solubilised by boiling in loading buffer prior to
14 discontinuous SDS polyacrylamide gel electrophoresis
15 (Laemmli (1970) Nature 277, 680-684) and immunoblotting
16 analysis (Khyse-Anderson (1984) Journal of Biochemical
17 and Biophysical Methods 10, 203-209). Human
18 α_1 -antitrypsin (AAT) was identified on immunoblot
19 filters by using goat-anti-AT serum [Protein Reference
20 Unit, Royal Hallamshire Hospital, Sheffield S10 2JF]
21 and anti-sheep/goat IgG serum conjugated to horseradish
22 peroxidase [Scottish Antibody Production Unit, Glasgow
23 and West of Scotland Blood Transfusion Service, Law
24 Hospital, Carlisle, Lanarkshire ML8 5ES].
25
26 Amounts of human α_1 -antitrypsin (AAT) in mouse milk
27 were measured by using LC-Partigen radial
28 immunodiffusion plates [Behring Diagnostics, Hoescht UK
29 Ltd, 50 Salisbury Road, Hounslow, Middlesex TW4 6JH].
30 The radial immunodiffusion (RID) method, which is
31 designed to detect AAT in body fluids in the
32 concentration range 8 - 125 μ g/ml, was carried out
33 according to the manufacturers instructions. Three

1 dilutions of standard human serum [LC-V, Behring
2 Diagnostics] were prepared in phosphate buffered saline
3 (PBS) to give AAT concentrations which fell within the
4 detection range for the assay.

5
6 Test milk samples were diluted 1:5 in distilled water
7 and defatted by spinning briefly in an Eppendorf 5415
8 centrifuge (BDH). The following control experiment was
9 carried out in order to assess the effect of the milk
10 environment on the detection of AAT (the method is
11 primarily designed for measuring AAT in blood serum).
12 Milk samples from non-transgenic mice were assayed with
13 and without defined amounts of added AAT. Samples
14 (20 μ l) were loaded into the wells and the plates left
15 open for 10 - 20 minutes. The plates were then sealed
16 with the plastic lids provided and left to stand at
17 room temperature. The diameters of the precipitation
18 zones were measured after a diffusion time of 2 - 3
19 days, using a low power binocular microscope fitted
20 with a lens graticule. At least three independent
21 readings were recorded and the average measurement (mm)
22 calculated and squared (mm^2). A calibration curve
23 plotting zone measurement squared against AAT
24 concentration was constructed using the values obtained
25 with the dilutions of standard human serum. This
26 linear graph was used to calculate the AAT
27 concentrations in the test samples.

28

29 Preparation of RNA

30

31 RNA may be prepared from mice immediately after milking
32 or from mice which have not been milked. The lactating
33 female mouse is killed by cervical dislocation and

1 tissues excised, taking care to avoid cross-
2 contamination of samples. The procedure is based on
3 the protocol described by Chirgwin, Przybyla, MacDonald
4 and Rutter (1979) Biochemistry 18, 5294-5299.
5
6 The tissue of interest is dissected and placed in 4 ml
7 of a 4 M solution of Guanadine Thiocyanate in a sterile
8 30 ml disposable plastic tube. The tissue is
9 homogenised using an Ultra-Turrax^R homogeniser at full
10 speed for 30 - 45 seconds at room temperature. The
11 homogenate is layered onto a 1.2 ml, 5.7 M CsCl
12 solution in a 5 ml polyallomer ultracentrifuge tube
13 (Sorvall Cat. 03127; Du Pont (UK) Ltd, Wedgwood Way,
14 Stevenage, Hertfordshire SG1 4QN, UK). The RNA is
15 pelleted through the cushion of CsCl by centrifuging at
16 36,000 rpm for 12 hrs at 20°C using a Sorvall AH650 or
17 Beckman SW50.1 swing-out rotor in a Beckman L80
18 ultracentrifuge (Beckman Instruments (UK) Ltd, Progress
19 Road, Sands Industrial Estate, High Wycombe, Bucks HP12
20 4JL, UK). After centrifugation the supernatant is
21 removed with sterile disposable plastic 5 ml pipettes
22 and the tube is then very carefully drained. The RNA
23 which should be visible as an opalescent pellet at the
24 bottom of the tube is resuspended in 2 ml of 7.5 M
25 Guanidine Hydrochloride with vigorous vortexing.
26 Resuspension may take 15 minutes or longer. The
27 preparation is transferred to a 15 or 30 ml
28 heat-sterilised CorexTM (Du Pont) centrifuge tube and
29 precipitated by the addition of 50 µl of 1M acetic acid
30 and 1ml of 100% ethanol and incubation overnight at
31 -20°C. The RNA is pelleted using a Sorvall SS34 rotor
32 (Du Pont) in a Sorvall RCB5 refrigerated centrifuge
33 (Du Pont) at 10,000 rpm for 10 minutes at 2°C. The RNA

1 pellet is resuspended in 2 ml of diethylpyrocarbonate
2 (Sigma) (DEPC)-treated distilled water by vortexing.
3 The RNA is re-precipitated by the addition of 600 μ l of
4 1M sodium acetate (DEPC-treated) and 3 volumes of 100%
5 ethanol, resuspended in DEPC treated water and again
6 precipitated. After the second precipitation from DEPC
7 water the RNA pellet is resuspended in DEPC water to
8 the desired final volume (usually 100 μ l - 500 μ l).
9 The concentration of RNA is determined spectro-
10 photometrically ($OD_{260nm} = 1$ corresponds to 40 μ g/ml).
11 RNA preparations are stored frozen at -70°C .

12

13 Analysis of RNA

14

15 The expression of the introduced transgene was
16 investigated in a number of different tissues by
17 'Northern' blotting of the RNA samples prepared by the
18 procedure described above. Aliquots (10 μ g-20 μ g) of
19 total RNA were denatured and separated in denaturing
20 MOPS/formaldehyde (1 - 1.5%) agarose gels and
21 transferred to HybondTM N (Amersham) nylon membranes as
22 described in the Amersham Handbook 'Membrane transfer
23 and detection methods' (PI/162/86/8 published by
24 Amersham International plc, PO Box 16, Amersham,
25 Buckinghamshire HP7 9LL, UK). The RNA bound to the
26 membranes is probed by hybridisation to appropriate ^{32}P
27 labelled DNA sequences (eg encoding BLG, FIX or AAT).
28 The labelling and hybridisation procedures are
29 described in section 1C above.

30

31 In some cases RNA transcripts were detected using an
32 RNase protection assay. This allows the determination
33 of the transcriptional start point of the gene. The

1 procedure essentially follows that described by Melton,
2 Krieg, Rebagliati, Maniatis, Zinn and Green (1984)
3 Nucleic Acids Research 18, 7035-7054. For example, for
4 FIX a 145bp SphI-EcoRV fragment from pSltgXSFIX
5 (WO-A-8800239) which spans the 5' fusion point of BLG
6 and FIX was cloned into SphI-SmaI cleaved pGEM4
7 (Promega Biotec, 2800 South Fish Hatchery Road,
8 Madison, Wisconsin 53791-9889, USA). A 192 nucleotide
9 long ^{32}P labelled, antisense RNA transcript was
10 generated using SP6 polymerase was used in the RNase
11 protection assays. After annealing the samples were
12 digested with RNAase A (BCL) (40 $\mu\text{g/ml}$) and RNase
13 T1(BCL) (2 $\mu\text{g/ml}$) at 37°C for one hour.
14 Phenol/Chloroform purified samples were electrophoresed
15 on 8% polyacrylamide/urea sequencing gels.

16

17 EXAMPLE 2: EXPRESSION OF THE AATB CONSTRUCT IN
18 TRANSGENIC MICE

19

20 The efficient expression of a human plasma protein in
21 the milk of transgenic mice is exemplified by construct
22 AATB. The details of the construction of AATB are
23 given in Example 1. Briefly AATB contains the genomic
24 sequences for the human (liver) α_1 -antitrypsin gene
25 minus intron 1, fused to the promoter of the ovine
26 beta-lactoglobulin gene. The fusion point is in the
27 5'-untranslated region of the BLG gene. It was
28 anticipated that the presence of the AAT introns would
29 enhance the levels of expression of the construct. The
30 large first AAT intron (ca. 5 kb) was omitted in order
31 to facilitate the DNA manipulation of the construct and
32 to determine whether all the AAT introns were required
33 for efficient expression.

1 Unless otherwise stated the analyses of expression are
2 tabulated. '+' indicates expression as determined by
3 the presence of the appropriate mRNA transcript
4 (detected by Northern blotting) or protein (as detected
5 by radial immunodiffusion (RID) or immunoblotting
6 (Western blotting)). '-' indicates that the expression
7 was not detected.

8

9 Transgenic mice carrying the AATB construct

10

11 The AATB construct described in Example 1 was used to
12 generate transgenic mice by the methods outlined in
13 Example 1. AATB construct DNA was microinjected into
14 fertilised mouse eggs on 7 occasions between August
15 1987 and June 1988. A total of 993 eggs were injected
16 of which 747 were transferred to recipient
17 pseudo-pregnant mice. A total of 122 pups were weaned.
18 Analysis of DNA prepared from tail biopsies, as
19 described in Example 1C, revealed that of these 122
20 generation zero (G0) pups 21 carried the AATB construct
21 as a transgene (see Table 1). These transgenic mice
22 had between 1 and >20 copies of the AATB construct
23 integrated into their genome.

24

25 The following policy was adopted for the study of the
26 expression of the AATB transgene. Where a founder
27 transgenic G0 individual was male, he was mated to
28 non-transgenic females to generate G1 offspring. Tail
29 DNAs from G1 individuals were examined to determine
30 whether they had inherited the transgene. Female
31 transgenic G1 mice were used for the analysis of
32 expression of the AATB transgene by the methods
33 described in Example 1D. Where a founder transgenic G0

1 individual was female she was used directly for the
 2 analysis of expression as described in Example 1D. The
 3 adoption of this policy meant that lines of mice were
 4 only established where the founder GO animal was male.
 5 The transmission of the transgenes to subsequent
 6 generations has also only been determined where the
 7 founder GO mouse was male. Transmission data for four
 8 AATB GO males is given in Table 1.

9
 10 TABLE 1: Mice carrying the AATB construct as a
 11 transgene.

14	Animal	Sex	Copy	Transmission data	
15	ID		Number	No. of offspring/No. transgenic	
17	AATB15	male	2-5	25	8
18	AATB17	male	10-15	26	16
19	AATB26	male	≥ 20	34	5
20	AATB28	male	2-5	22	12
21	AATB44	female	15		
22	AATB45	female	1-2		
23	AATB65	female	2-3		
24	AATB69	female	1-2		
25	AATB105	female	20		

26
 27 Analysis of expression

28
 29 Fifteen G1 females have been examined for expression of
 30 the AATB transgene, 8 by protein analysis of milk and 7
 31 by RNA analysis by the methods described in Example 1.
 32 A further 5 GO females have been examined by both
 33 protein analysis of milk and RNA analysis. A total of

1 9 different transgenic mice or mouse-lines were
2 examined.

3

4 RNA Analysis

5 RNAs isolated from the following tissues were examined
6 for the presence of AATB transcripts - mammary gland,
7 liver, kidney, spleen, salivary gland and heart. Total
8 RNA samples (10 µg) from these tissues were analysed by
9 Northern blotting. A representative Northern blot is
10 presented as Figure 11 [Lanes 1 & 2, and 3 & 4 contain
11 mammary (M) and liver (L) samples from control mice;
12 lanes 5 - 9, AATB26.1 mammary (M), liver (L), kidney
13 (K), spleen (Sp) and salivary (Sa) RNA samples; lanes
14 10 - 14, AATB17.3 mammary (M), liver (L), kidney (K),
15 spleen (Sp) and salivary (Sa) RNA samples. The AAT
16 transcript of approximately 1400 nucleotides is
17 arrowed]. The human AAT cDNA probe, p8α1ppg,
18 cross-hybridises with endogenous mouse AAT transcripts
19 in liver RNA samples. The presence of AAT transcripts
20 in salivary samples from AATB26.1 and AATB17.3 do not
21 result from contamination with liver or mammary
22 material as proved by re-probing the filters with
23 liver-specific and salivary-specific probes. The
24 results of this analysis are summarised in Table 2.

25

26

27

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1 TABLE 2: Summary of RNA analysis for AATB transgenic
2 mice.

3	4 Animal ID	5 Generation	6 Tissue (presence/absence of AATB transcripts)					
			Mam.	Liver	Kid.	Spl.	Saliv.	Heart
7	AATB15.2	G1	+	*	?	-	-	-
8	AATB15.13	G1	-	?	-	-	-	NT
9	AATB17.3	G1	+	?	-	-	+	NT
10	AATB17.20	G1	+	-	-	-	+	NT
11	AATB26.1	G1	-	-	-	-	+	NT
12	AATB26.28	G1	-	?	-	-	+	-
13	AATB28.3	G1	-	?	-	-	-	NT
14	AATB28.21	G1	-	?	-	-	-	NT
15	AATB44	GO	+	?	-	-	-	-
16	AATB45	GO	+	?	-	-	-	-
17	AATB65	GO	+	?	-	-	-	-
18	AATB69	GO	+	?	-	-	-	-
19	AATB105	GO	-	?	-	-	+	-

20

21 [Mam. = mammary gland; Kid. = kidney; Spl. = spleen;
22 Saliv. = salivary gland; nd = not detected; NT = not
23 tested]

24 * presence only detected in poly A+ RNA

25 ? background from endogenous mouse AAT transcripts in
26 liver precluded an unambiguous determination of whether
27 there were AATB transcripts present.

28

29 In order to confirm that the transcripts observed were
30 being initiated at the beta-lactoglobulin start site in
31 the AATB constructs, RNAs isolated from the mammary
32 gland of mouse AATB17.20 and from the salivary gland of
33 mouse AATB26.1 were examined by an RNase protection

1 assay as described in Example 1D. RNAs isolated from
2 the liver (AATB17.20 & AATB26.1) and from the mammary
3 gland (AATB26.1) of these mice were also examined by
4 RNase protection, as were RNAs from non-transgenic
5 liver, mammary gland and salivary gland. The
6 anti-sense probe was produced by transcribing a pGEM
7 vector (Promega Biotec, 2800 South Fish Hatchery Road,
8 Madison, Wisconsin 53791-9889) containing a 155 bp SphI
9 - BamHI fragment derived from the 5' end of the AATA
10 construct. This 155 bp fragment is identical to that
11 found in AATB (see pIII-ISpB, Example 1A). Annealing
12 was carried out under standard conditions and the
13 hydrolysis of single-stranded RNA performed with RNaseA
14 and RNaseT1(BCL). A sense transcript was also
15 transcribed and various amounts of this transcript
16 included along with 20 µg samples of control RNA to
17 provide an estimation of steady state mRNA levels. A
18 representative RNase protection gel is shown in Figure
19 12 [Lanes 1 & 2, AATB17.20 20 µg and 10 µg total
20 mammary RNA; lanes 3, 4, 5 & 6, 1000 pg, 200 pg, 100 pg
21 & 50 pg of control sense transcript; lanes 7 & 8,
22 AATB26.1 20 µg & 10 µg total salivary RNA; lanes 9, 10
23 & 11, 5 µg aliquots of mammary polyA+ RNA from
24 AATB15.2, AATA5.20 and AATA31; lane M HaeIII digested
25 ΦX174 DNA marker track]. The RNase protection assay
26 confirmed that the beta-lactoglobulin transcription
27 start site was being used as predicted in the mammary
28 tissue of line AATB17 and in the salivary tissue of
29 line AATB26. The absence of AATB transcripts in the
30 liver of AATB17.20 and in the liver and mammary gland
31 of AATB26.1 were also confirmed by RNase protection
32 assays.

33

1 Protein analysis of milk
2 Milk samples from 8 G1 females and from 5 G0 females
3 were assayed for the presence of human
4 α_1 -antitrypsin by the immunoblotting methods
5 described in Example 1D. The results of this analysis
6 are summarised in Table 3. A representative immunoblot
7 of diluted milk samples from transgenic and normal mice
8 is shown as Figure 13 [lanes 1, pooled human serum; 2,
9 control mouse milk; 3, AATB 15.10 milk; 4, AATB 17.24
10 milk; 5, AATB 17.23 milk; 6, AATB 15.20 milk; 7,
11 control mouse milk; 8 & 9, marker proteins]. The human
12 AAT (arrowed) is clearly evident in preparations from
13 mice AATB17.23 and AATB17.24 and just about visible in
14 milk from mouse AATB15.10]. Cross reaction of the
15 anti-human sera to endogenous mouse AAT (which migrates
16 slightly faster than its human counterpart) is also
17 evident.

18

19 Amounts of human α_1 -antitrypsin in transgenic mouse
20 milk were estimated using LC-Partigen radial
21 immunodiffusion plates [RID] [Behring Diagnostics,
22 Hoescht UK Ltd, 50 Salisbury Road, Hounslow, Middlesex
23 TW4 6JH] as described in Example 1D (see Table 3).
24 Normal mouse milk samples with and without human
25 α_1 -antitrypsin were included as controls.

26

27

28

29

30

31

32

33

1 TABLE 3

2	3	4	5	6
	Animal	Generation	Immunoblot	RID
	ID		presence/absence	protein mg/ml
6	AATB15.10	G1	+	NT
7	AATB15.20	G1	-	NT
8	AATB17.23	G1	+	0.448
9	AATB17.24	G1	+	0.533
10	AATB26.14	G1	-	NT
11	AATB26.28	G1	-	NT
12	AATB28.11	G1	-	NT
13	AATB28.14	G1	-	NT
14	AATB44	GO	+	0.87
15	AATB45	GO	+	0.088
16	AATB65	GO	+	0.091
17	AATB69	GO	+	0.465
18	AATB105	GO	-	-

19
20 [NT = not tested]

21
22 Of the nine different AATB transgenic mice or
23 mouse-lines examined, five efficiently directed
24 expression of human α_1 -antitrypsin in milk. A
25 sixth line (AATB15) also exhibited mammary expression,
26 but at lower levels. This analysis proves that the
27 AATB construct contains sufficient information to
28 direct efficient expression of human α_1 -antitrypsin
29 in the mammary glands of transgenic mice. There
30 appears to be some relaxation of the tissue-specificity
31 of the BLG promoter such as to allow it to function in
32 salivary gland as well as in the mammary gland. The
33 first intron of the AAT gene is not necessary for

1 efficient expression of the hybrid gene AATB. The
2 introns and 3' flanking sequences of the BLG gene are
3 evidently not essential for efficient mammary gland
4 expression from the BLG promoter. The 5' flanking
5 sequences of the BLG gene from SalI through SphI to the
6 PvuII site in the 5'-untranslated of the BLG gene are
7 sufficient to direct the efficient mammary expression
8 of a heterologous gene as exemplified by AAT.

9

10 **EXAMPLE 3 : COMPARATIVE EXPRESSION OF BLG CONSTRUCTS**

11

12 The efficient expression of a human plasma protein in
13 the milk of transgenic mice is exemplified by construct
14 AATB. In this section the expression analyses of
15 different constructs encoding a human plasma protein,
16 either FIX or AAT, are given. The details of their
17 constructions are given in Example 1A. Expression
18 analyses of two configurations of the BLG gene are also
19 given and serve to further define the BLG sequences
20 that may be required for expression in the mammary
21 gland. Unless otherwise stated the analyses of
22 expression are tabulated. '+' indicates expression as
23 determined by the presence of the appropriate mRNA
24 transcript (detected by Northern blotting) or protein
25 (as detected by radioimmunoassay (RIA), radial-
26 immunodiffusion (RID), Coomassie blue staining or
27 Western blotting. '-' indicates that expression was
28 not detected.

29

30 **FIXA:**

31

32 Construction and expression of this construct is
33 described in detail in WO-A-8800239 (designated

1 pSS1tgXS-FIX or pSS1tgXS-TARG). It comprises cDNA
 2 sequences encoding human blood clotting factor IX (FIX)
 3 inserted into the first exon of the BLG gene.
 4 Transgenic sheep have been produced which carry this
 5 construct and these have been analysed for the
 6 expression of human FIX by Northern blotting of mammary
 7 RNA and radioimmunoassays of milk:-

8				
9	Sheep	Description	RNA	FIX Protein (iu*/l)
10	6LL240	GO female	+	+: 4.7 ^a , 8.0 ^b
11	6LL231	GO female	+	+: 4.0a, 4.3b
12	7R45	G1 female@	+	+: / 5.7b
13	7R39	G1 female@	+	+: / 6.4b

14
 15 [a, analysis by RIA in 1987; b, analysis in 1988;
 16 *, 1 iu = 5 µg; @, daughters of transgenic male 6LL225]

17
 18 The human FIX protein in transgenic sheep milk has been
 19 visualised by Western blotting and also shown to have
 20 biological activity. However, the level of protein in
 21 the milk is far below that necessary for commercial
 22 exploitation.

23

24 AATA:

25

26 This construct comprises the cDNA encoding human AAT
 27 inserted into the first exon of the BLG gene. It is
 28 equivalent to FIXA and thus can be considered as an
 29 example of the generalised construct designated
 30 pSS1tgXS-TARG as described in WO-A-8800239. It has
 31 been used to produce transgenic sheep and mice.

32

33

1	Sheep	Description	RNA	AAT Protein*
2	6LL273	GO female	-	-
3	6LL167	GO female	nd	+ (2-10 μ g/ml)
4	7LL183	GO female	nd	nd

5 *protein detected and estimated by Western blotting of
6 milk samples
7 nd; not done

8
9 Western blots of milk whey samples from normal and the
10 two transgenic sheep analysed are shown in Figure 14
11 [lanes 1, 7LL167(AATA); 2, control sheep whey; 3, human
12 serum pool; 4, 7LL167(AATA); 5, 6LL273(AATA); 6,
13 control sheep whey].

14
15 The human AAT (arrowed) is clearly evident in milk whey
16 samples from 6LL167 but is not present in that from
17 6LL273 or control sheep milk. Under these conditions
18 endogenous AAT present in sheep milk is detected by the
19 anti-human sera and has a greater electrophoretic
20 mobility than its human counterpart.

21
22 The levels of human AAT estimated to be present in the
23 transgenic sheep milk are low and are not sufficient
24 for commercial exploitation.

25
26 Expression of the AATA construct has also been studied
27 in transgenic mice.

28
29
30
31
32
33

1	Mice	Description	RNA	AAT protein*
2	AATA1.5	line segregating	-	-
3		from AATA1		
4	AATA1.8	line segregating		
5		from AATA1	+	+ (<<2 μ g/ml)
6	AATA5	mouse-line	+	+ (2-10 μ g/ml)
7	AATA31	mouse-line	-	-

8 *AAT protein detected and estimated by Western
9 blotting.

10

11 Western blots of TCA precipitated whey samples from
12 normal and transgenic mice are shown in Figure 15
13 [Lanes 1, human α_1 -antitrypsin antigen (Sigma); 2,
14 human serum; 3, mouse serum; 4, AATA 1.8.1 whey; 5,
15 AATA 1.5.10 whey; 6, human and mouse serum; 7, control
16 mouse whey; 8, AATA 5.30 whey; 9, AATA 1 whey; 10,
17 human serum; 11, mouse serum]. The human AAT (arrowed)
18 is clearly evident in preparations from mouse-line
19 AATA5 and is just about visible in mouse-line AATA1.8.
20 Cross-reaction of the anti-human sera with endogenous
21 mouse AAT (which migrates slightly faster than its
22 human counterpart) is also evident.

23

24 The levels of expression observed in mouse-line AATA5
25 are of the same order of magnitude as is observed in
26 transgenic sheep 7LL167, and as such would not prove
27 commercial even if obtained in a dairy animal such as a
28 sheep.

29

30 BLG-BLG

31

32 This construct comprises the BLG cDNA inserted into
33 exon1 of the BLG structural gene. The construct is

1 analogous to AATA and FIXA (ie pSSltgXS-TARG) in that
2 the complete structural gene of BLG is present as well
3 as the cDNA ins rt. In this case, however, the insert
4 is a cDNA encoding a milk protein, rather than a cDNA
5 from a gene normally expressed in another tissue. The
6 expression of this construct was assessed in transgenic
7 mice.

8

9 Mice	Description	RNA	BLG protein*
10 BB4	GO female	+	+(<.005mg/ml)
11 BB5	GO female	+	+(<.005mg/ml)
12 BB19	GO female	+	+(<.005mg/ml)
13 BB47	GO female	+	+(<.005mg/ml)
14 BB55	GO female	nd	+(<.005mg/ml)

15 *detected and estimated by Western blotting
16 nd = not determined

17
18 The construct was expressed tissue-specifically in the
19 four mice in which RNA was analysed. In all five
20 animals low levels of BLG were detected in the milk.
21 These levels of BLG are far below that observed with
22 expression of the normal structural BLG gene (eg see
23 Example 7 in WO-A-8800239). The data show that the
24 'A-type' construct even when encoding a natural milk
25 protein gene such as BLG (which is known to be capable
26 of very high levels of expression in the mammary gland)
27 is not expressed efficiently in the mammary gland of
28 transgenic mice. This suggests that it may be the
29 configuration of cDNA (whether FIX, AAT or BLG) with
30 the genomic BLG sequence (ie insertion into the first
31 exon) which is responsible for the low levels of
32 expression of this type of construct.

33

1 AATD**2**

3 This construct comprises the AAT cDNA fused to 5' BLG
4 sequences and with 3' sequences from exons 6 and 7 of
5 BLG and the 3' flanking sequences of the BLG gene.
6 This gene contains no introns. Its potential for
7 expression was assessed in transgenic mice:-

8

9 Mice	Description	RNA	AAT Protein*
10 AATD12	GO female	-	-
11 AATD14	GO female	-	-
12 AATD31	GO female	-	-
13 AATD33	GO female	-	-
14 AATD9	mouse-line	-	-
15 AAT21	mouse-line	-	-
16 AATD41	mouse-line	-	-
17 AATD47	mouse-line	-	-

18 *assessed by Western blotting

19

20 None of the transgenic mice carrying AATD expressed the
21 transgene.

22

23 FIXD This is an analogous construct to AATD and
24 comprises the FIX cDNA sequences fused to BLG 5' and 3'
25 sequences (including exons 6 and 7) and contains no
26 introns. Expression was assessed in transgenic mice.

27**28****29****30****31****32****33**

	Mice	Description	RNA	FIX Protein*
1	FIXD11	GO f male	-	-
2	FIXD14	GO female	-	-
3	FIXD15	GO female	-	-
4	FIXD16	GO female	-	-
5	FIXD18	GO female	-	-
6	FIXD20	mouse-line	-	-
7	FIXD23	mouse-line	-	-
8	FIXD24	mouse-line	-	-
9	FIXD26	mouse-line	-	-

10 *assessed by Western blotting

11

12
13 None of the transgenic mice carrying FIXD expressed the
14 transgene.

15

16 These data, together with those from AATD, suggest that
17 a simple configuration of BLG 5' and 3' sequences and
18 target cDNA sequences (ie FIX or AAT) in which no
19 introns are present in the construct will not be
20 expressed efficiently, if at all, in the mammary gland
21 of a transgenic animal.

22

23 AATC

24

25 This construct comprises the AAT cDNA inserted into the
26 second exon of BLG. It was constructed to determine
27 whether or not inserting the target cDNA (in this case
28 AAT) at a site distant from the promoter (ie in the
29 second rather than in the first exon) would improve the
30 levels of expression. Expression was assessed in
31 transgenic mice.

32

33

1	Mice	D scription	RNA	AAT Protein*
2	AATC14	GO female	-	-
3	AATC24	GO female	-	-
4	AATC25	GO female	-	-
5	AATC30	GO female	-	-
6	AATC4	mouse-line	+	-
7	AATC5	mouse-line	-	-
8	AATC27	mouse-line	-	-

9 *assessed by Western blotting

10

11 Only one out of seven 'lines' expressed the transgene
 12 as determined by RNA; in this line no AAT protein was
 13 detected, presumably because re-initiation from the
 14 initiating ATG of the AAT sequences did not occur. In
 15 the RNA-expressing line expression appeared to occur
 16 only in the mammary gland although at low levels.
 17 These data would suggest that moving the site of
 18 insertion of the target cDNA to the second exon (and
 19 thus including intron 1 of the BLG) does not yield
 20 improved levels of expression of the target cDNA (in
 21 this case AAT).

22

23 DELTA A2

24

25 This construct contains the minimum ovine BLG sequences
 26 that have so far been shown in transgenic mice to be
 27 required for efficient and tissue-specific expression
 28 of BLG in the mammary gland. It is a 5' deletion
 29 derivative of pSS1tgXS (WO-A-8800239) and has only
 30 799 bp of sequence flanking the published mRNA cap site
 31 (Ali and Clark, (1988) J. Mol. Biol. 199, 415-426).
 32 This deleted version of pSS1tgXS has been used to
 33 produce transgenic mice.

1	Mouse	Description	RNA	BLG Protein*
2	DELTA A2/1	GO female	+	+ ~2mg/ml
3	DELTA A2/28	GO female	+	+ ~3mg/ml
4	DELTA A2/38	GO female	+	+ <0.15mg/ml

5

6 Detected by Coomassie blue staining: estimated
7 densitometrically.

8

9 The DELTA A2 constructs shows that 799 bp of 5'
10 flanking sequences are sufficient for correct and
11 efficient expression of BLG in the mammary gland of
12 transgenic mice. This construct also contains the
13 4.9kb transcription unit of BLG and 1.9kb of 3'flanking
14 sequences. It is conceivable that important regulatory
15 sequences for mammary expression are present in these
16 regions. (However, note the result with AATB in which
17 these sequences were absent and yet efficient mammary
18 expression was obtained.)

19

20 EXAMPLE 4 : PREPARATION OF FACTOR IX CONSTRUCT

21

22 Strategy

23

24 The expression in transgenic sheep of a human Factor IX
25 gene, called BLG-FIX, is disclosed in WO-A-8800239 and
26 Clark et al (1989) (Biotechnology, 7 487-492), both of
27 which are herein incorporated by reference, insofar as
28 the law allows. Since this construct has been
29 previously referred to as FIX A, this nomenclature is
30 retained. Essentially the FIX A construct comprises
31 the insertion of a human FIX cDNA into the first intron
32 of the complete (ie all exons and introns present)
33 sheep betalactoglobulin (BLG) gene. This example

1 relates to the modification of this FIX A construct to
2 the effect that the first intron of the human genomic
3 FIX gene has been inserted at the appropriate position,
4 into the FIX cDNA, so that on transcription of the new
5 gene, a primary transcript containing an intron will be
6 produced. When this transcript is correctly spliced, a
7 transcript will be generated, which on translation,
8 will generate exactly the same protein as the original
9 FIX A construct.

10

11 The construction route shown below is complicated, but
12 the methods used are as described in Example 1. The
13 difficulties were caused by the size of human FIX
14 genomic DNA fragments and the requirement to develop
15 new shuttle vectors to allow the suitable manipulation
16 of the BLG and FIX DNA sequences.

17

18 A.

19 Aims

20 Construction of -

21

- 22 a) pUC PM - modified cloning vector.
23 b) pUC XS - pUC PM containing BLG genomic DNA.
24 c) pUC XS/RV - pUC XS containing a unique EcoRV
25 restriction site in the BLG 5'
26 untranslated region.

27

28 Details

29

- 30 i A double stranded synthetic linker DNA including
31 in the following order the restriction sites for
32 the enzymes EcoRI, PvuI, MluI, SalI, EcoRV, XbaI,
33 PvuI, MluI, HindIII (see Fig 16a) was ligated into

- 1 EcoRI/HindIII digested, gel purified, pUC 18
2 (Boehringer) to generate pUC PM (see Fig 16a).
3 The insertion was checked by both restriction
4 analysis and direct sequencing.
5
- 6 ii A SalI-XbaI fragment purified from pSSltgXS (this
7 contains the XbaI-SalI BLG genomic fragment in
8 pPOLY III.I (see Figure 3 of WO-A-8800239) was
9 ligated into SalI/XbaI digested, CIP (calf
10 intestinal phosphatase) (see Fig 16a) - treated,
11 gel purified, pUC PM to give pUC XS. This was
12 checked by restriction analysis.
13
- 14 iii A synthetic EcoRV linker
15
16 (5' TCGACGCGGCCGCGATATCCATGGATCT)
17 (GCTGCGCCGGCGCTATAGGTACCTAGAGATC 5')
18
- 19 was ligated into the unique PvuII site of
20 PvuII-digested pSSltgSE (see WO-A-8800239 -
21 pSSltgSE comprises a SphI-EcoRI fragment of BLG
22 inserted into pPOLY III.I; the PvuII site is 30
23 bases downstream of cap site in the first exon of
24 BLG) - see Fig 16b.
25
- 26 iv The SphI-NotI fragment containing the EcoRV linker
27 was gel purified from pSSltgSE/RV and ligated into
28 the SphI, NotI digested, CIP - treated, gel
29 purified pUC XS, generating pUC XS/RV - see Fig
30 16b.
31
- 32 This was checked by restriction analysis.
33

1 B.

2 Aims

3 Construction of -

4

5 a) Clones 9-3, B6 and 9 H11 - cloning vehicles from
6 transfer of various portions of FIX genomic DNA.

7

8 b) Clone 11-6, this comprises exons 1, 2, 3 and
9 introns 1, 2 of FIX inserted into pUC 9.

10

11 Details

12

13 i Cosmid clone cIX2, containing part of FIX gene,
14 was obtained from G. Brownlee (see GB-B-2125409,
15 also P.R. Winslip, D. Phil Thesis, Oxford, and
16 Anson et al (1988) EMBO J. 7 2795-2799).

17

18 Note In the following description - the assignment of a
19 base number to a restriction site refers to the
20 number of bases the site is upstream (mins sign)
21 or downstream of the cap site in the first FIX
22 exon. These numbers are obtained by analogy, from
23 the published FIX sequence of Yoshitake et al
24 (1985) Biochemistry 24 3736-3750.

25

26 ii Clone 9-3 was produced by ligating gel purified
27 BamHI (-2032) - EcoRI (5740) fragment from cIX2
28 into BamHI/EcoRI-digested, CIP-treated, gel
29 purified, pUC 9 (see Fig 17).

30

31 iii Clone 9 H11 was made by ligating the gel purified
32 HindIII (810) - HindIII (8329) fragment from cIX2
33 into HindIII-digested, CIP-treated, gel purified
34 pUC 9 (see Fig 17).

- 1 iv Clone 9-3 was digested with BamHI and HpaI, end
2 filled with the Klenow enzyme, and the large
3 fragment was gel purified and ligated to produce
4 clone B6 (see Fig 17). The net effect of this is
5 to remove the FIX sequence between -2032 and -830.
6
7 v Clone 9H 11 was digested with SalI and BglII,
8 CIP-treated and then the large fragment, now
9 lacking the regions between the vector SalI site
10 and the FIX BglII site (3996) was gel purified.
11 This was ligated with the gel purified SalI
12 (vector) - BglII (3996) fragment from clone B6, to
13 generate clone 11-6 (see Fig 17) which contains
14 FIX sequence -830 - -8329 (ie exons 1,2,3 introns
15 1,2).

16
17 C.

18 Aims

19 Construction of -

- 20
21 a) Clone C8 (incorporating 5' portion of FIX cDNA).
22 b) Clone C81.SK (incorporating 5' portion of FIX cDNA
23 + FIX intron I).
24

25 Details

- 26
27 i FIX A (FIX cDNA in BLG gene, called BLG FIX in
28 Clark et al, (1989) Biotechnology 7 487-492, also
29 see WO-A-8800239) was digested with Sph I/Bst Y 1.
30 The small fragment was gel purified and ligated
31 into SphI/BamHI-digested, CIP-treated, pUC 18
32 (Boehringer) generating clone C8 (see Fig 18) DNA
33 was prepared by growth in a dam⁻ E. coli host (SK
34 383) to allow Bcl digestion.

1 Note C8 contains most of FIX cDNA and 2 out of 3 BclI
2 sites (at positions 2 and 81 upstream of the first
3 nucleotide of the first AUG of the FIX cDNA
4 sequence shown in Fig 9, GB-B-2125409; these are
5 equivalent to Bcl sites 46 (exon 1) and 6333 (exon
6 2) of genomic DNA.

7

8 ii C8 was digested with BclI, CIP-treated and the
9 large fragment retained after gel purification.

10

11 iii Clone 11-6 DNA was prepared from E. coli host SK
12 383 (dam⁻) and the 6287 bp BclI fragment
13 containing intron 1 purified and ligated with the
14 large C8 fragment described in ii above, to
15 generate C81 SK - see Fig 18. The Bcl junctions
16 were sequenced to confirm reconstruction of Bcl
17 sites.

18

19 4.

20 Aims

21 Construction of -

22

23 a) J FIX A (FIX A insert transferred to pUC PM).

24 b) SP FIX (A cloning vehicle for transfer of intron 1
25 to J FIX A).

26

27 Details

28

29 i SphI-NotI fragment from FIX A, containing FIX cDNA
30 and flanking BLG sequence was gel purified and
31 ligated into SphI/NotI digested, CIP-treated, gel
32 purified pUC XS/RV to generate J FIX A (see Fig
33 19).

1 ii Sph-NruI fragment containing FIX cDNA from J FIX A
2 was gel purified and ligated into SphI/EcoRV
3 digested, CIP treated, pSP 72 (promega Biotech) to
4 generate SP FIX (see Fig 19).

5

6 E.

7 Aims

8 Construction of -

9

- 10 a) b 11 - cloning vehicle containing FIX intron 1.
11 b) J FIX A 1 - final "minigene" construct for
12 construction of transgenics.

13

14 Details

15

16 i SP FIX and C81.SK digested to completion with
17 SphI, then partially digested with Ssp 1*. A 7.2
18 kb fragment from C81.SK containing FIX intron 1
19 was ligated with the CIP-treated, gel purified
20 large fragment of SP FIX to generate clone b 11
21 (see Fig 20) which contains the complete FIX cDNA
22 and FIX intron 1.

23

24 ii The SphI-NotI fragment from b11 containing the FIX
25 sequences was gel purified and ligated into
26 SphI/NotI digested, CIP-treated J FIX A to
27 generate J FIX A 1 (see Fig 20).

28

29 *Note - In SP FIX, there is a SspI site in vector which
30 was not excised in the partially digested fragment
31 shown. Likewise in C81.SK there are four SspI
32 sites in the FIX intron. The 7.2K fragment
33 contains all these four sites and in fact

1 terminates at the SspI site at position 30830 b of
2 the genomic FIX sequence.

3

4 F.

5

6 Transgenic mice were constructed as described in
7 Example 1B, and identified as described in Example 1C.
8 One male and one female transgenic mice were initially
9 identified.

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1 CLAIMS

2

3 1. A genetic construct comprising a 5' flanking
4 sequence from a mammalian milk protein gene and DNA
5 coding for a heterologous protein other than the milk
6 protein, wherein the protein-coding DNA comprises at
7 least one, but not all, of the introns naturally
8 occurring in a gene coding for the heterologous protein
9 and wherein the 5'-flanking sequence is sufficient to
10 drive expression of the heterologous protein.

11

12 2. A construct as claimed in claim 1, wherein the
13 milk protein gene is a beta-lactoglobulin gene.

14

15 3. A construct as claimed in claim 2, including about
16 800 base pairs upstream of the beta-lactoglobulin
17 transcription start site.

18

19 4. A construct as claimed in claim 2, including about
20 4.2 kilobase pairs upstream of the beta-lactoglobulin
21 transcription start site.

22

23 5. A construct as claimed in claim 1, wherein the
24 heterologous protein is a serine protease.

25

26 6. A construct as claimed in claim 2, wherein the
27 heterologous protein is a blood factor.

28

29 7. A construct as claimed in claim 1, in which all
30 but one of the natural introns are present.

31

32 8. A construct as claimed in claim 1, in which only
33 one of the natural introns are present.

1 9. A construct as claimed in claim 1 comprising a
2 3'-sequence.

3

4 10. A method for producing a substance comprising a
5 polypeptide, the method comprising introducing a DNA
6 construct as claimed in claim 1 into the genome of an
7 animal in such a way that the protein-coding DNA is
8 expressed in a secretory gland of the animal.

9

10 11. A method as claimed in claim 10, wherein the
11 animal is a mammal and the secretory gland is a mammary
12 gland.

13

14 12. A vector comprising a genetic construct as claimed
15 in claim 1.

16

17 13. A cell containing a vector as claimed in claim 12.

18

19 14. An animal cell comprising a construct as claimed
20 in claim 1.

21

22 15. A transgenic animal comprising a genetic construct
23 as claimed in claim 1 integrated into its genome.

24

25 16. A transgenic animal as claimed in claim 15 which
26 is capable of transmitting the construct to its
27 progeny.

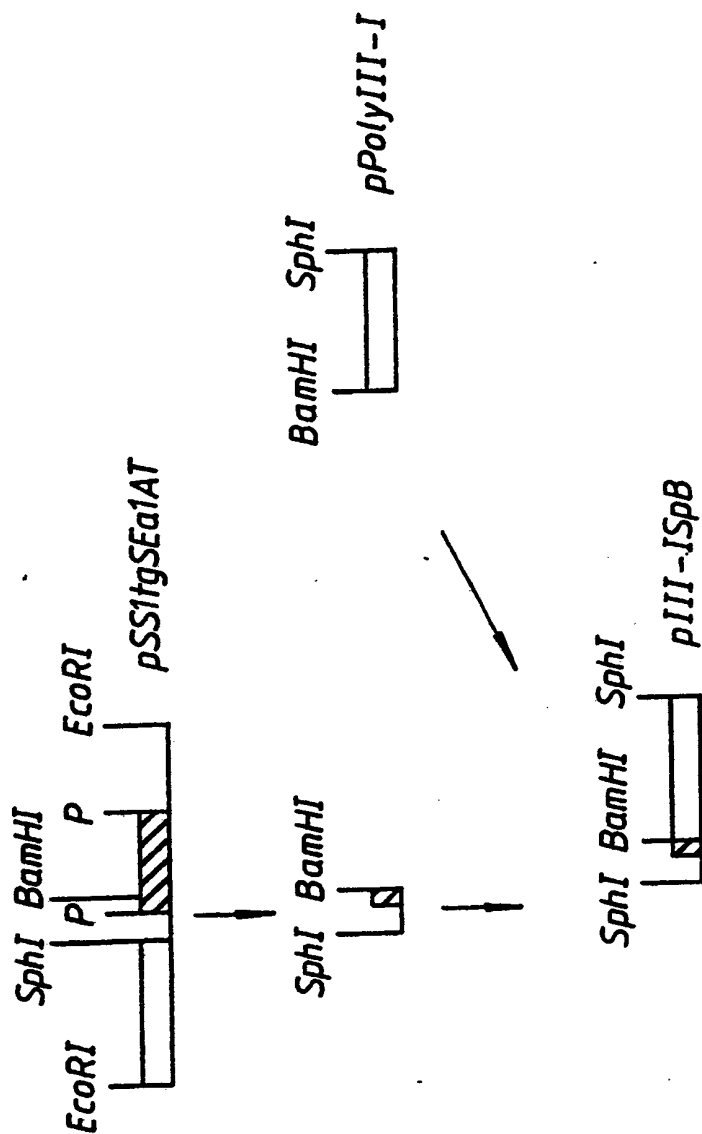
28


29 17. A method for producing a substance comprising a
30 polypeptide, the method comprising harvesting the
31 substance from a transgenic animal as claimed in claim

32 15.


33

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 alpha1-antitrypsin cDNA sequences

 plasmid sequences

 beta-lactoglobulin sequences


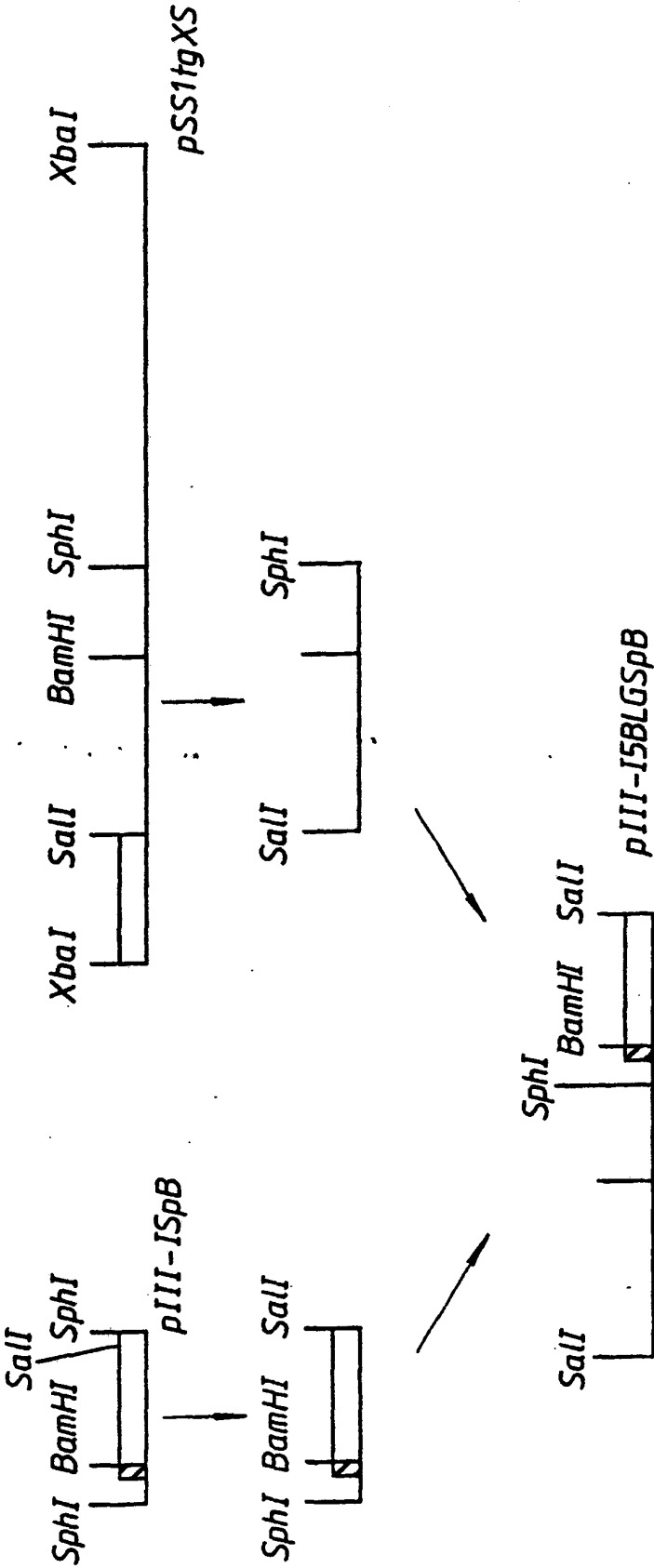
 P=PvuII-0 sites

Fig.1.

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alpha1-antitrypsin cDNA sequences

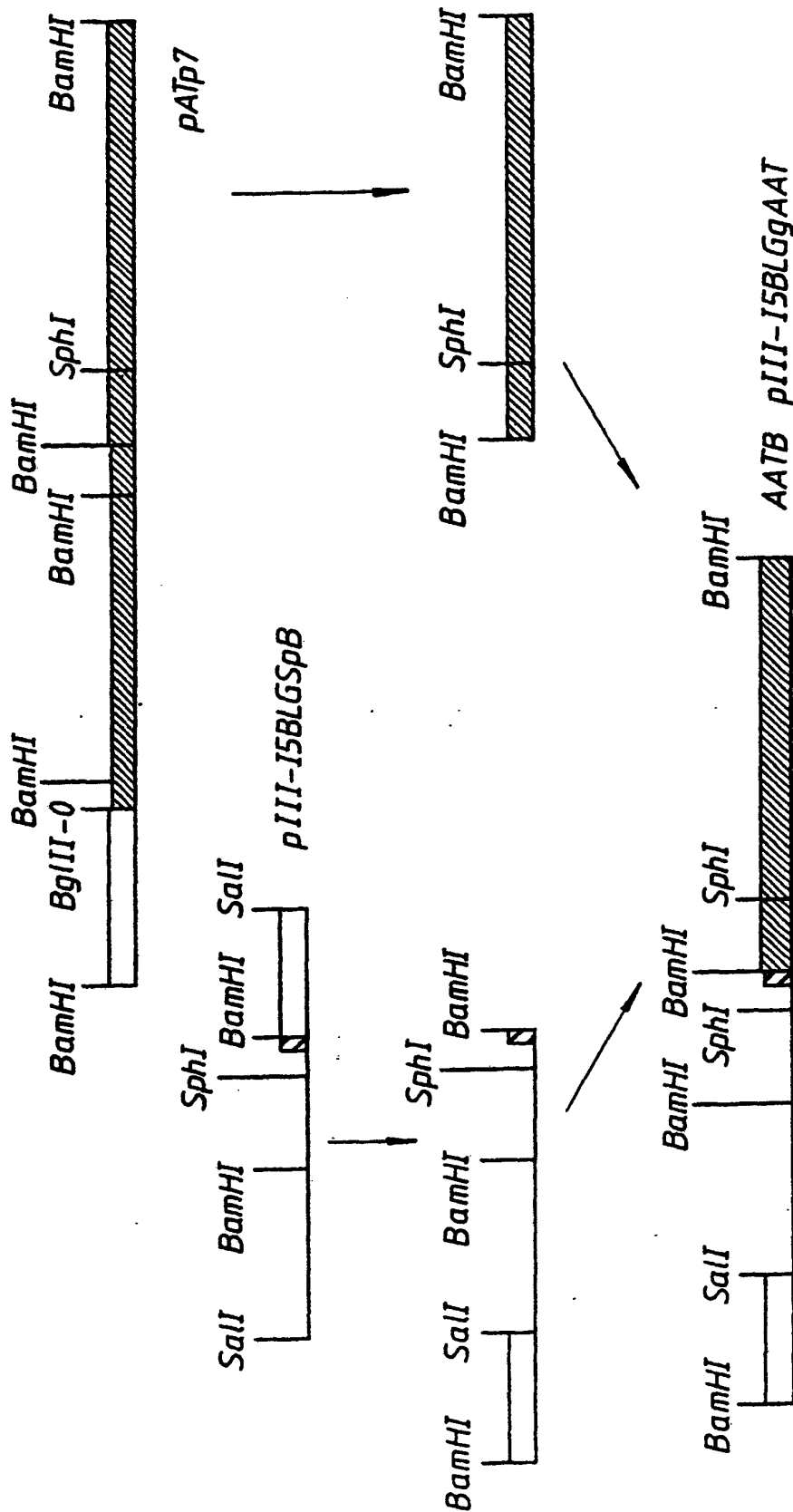
plasmid sequences



beta-lactoglobulin sequences

note: not all BamHI sites are shown for pSS1tgXS

Fig.2.

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 alpha1-antitrypsin cDNA sequences
 alpha1-antitrypsin genomic sequences



 plasmid sequences
 beta - lactoglobulin sequences

Fig.3.

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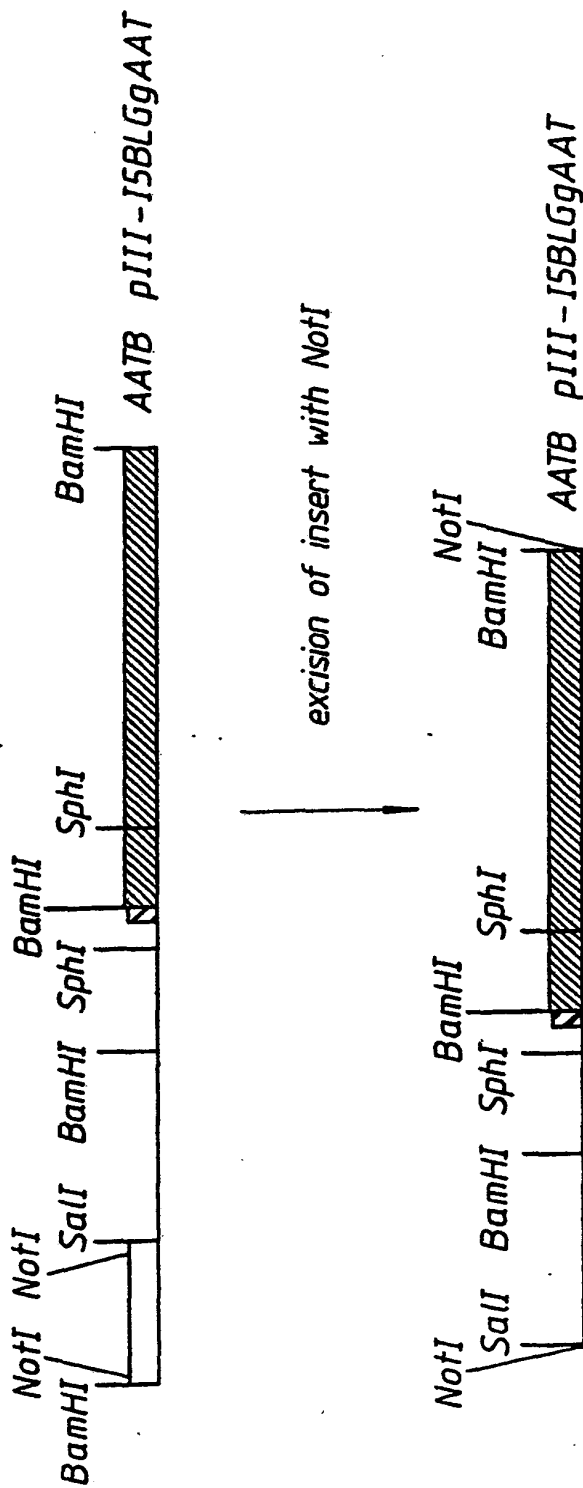


Fig.4.

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SphI

gcatgcgccctcctgtataaggccccaagcctgctgtctcagccctcc

BLG|AAT

*->

actccctgcagagctcagaagcagacccag|cgacaatgccgtcttct
PvuII-0 | TaqI-0 MetProSerSerValSerTrpGlyIleLeuLeuLeuAlaGlyLeuCysCysLeuValPro
gtctcgtggggcatcctcctgctggcaggcctgtgctgcctgggtccct
^ ^ ^

BamHI

ValSerLeuAlaGluAspProGlnGlyAsp
gtctccctggctgaggatccccaggagat

Sequence of AATB (pIII-15BLGgAAT) from the SphI site corresponding to the 5' flanking sequences of β -lactoglobulin through the fusion to the alpha₁-antitrypsin sequences. The key restriction sites for SphI and BamHI are underlined.

** = transcription start point*

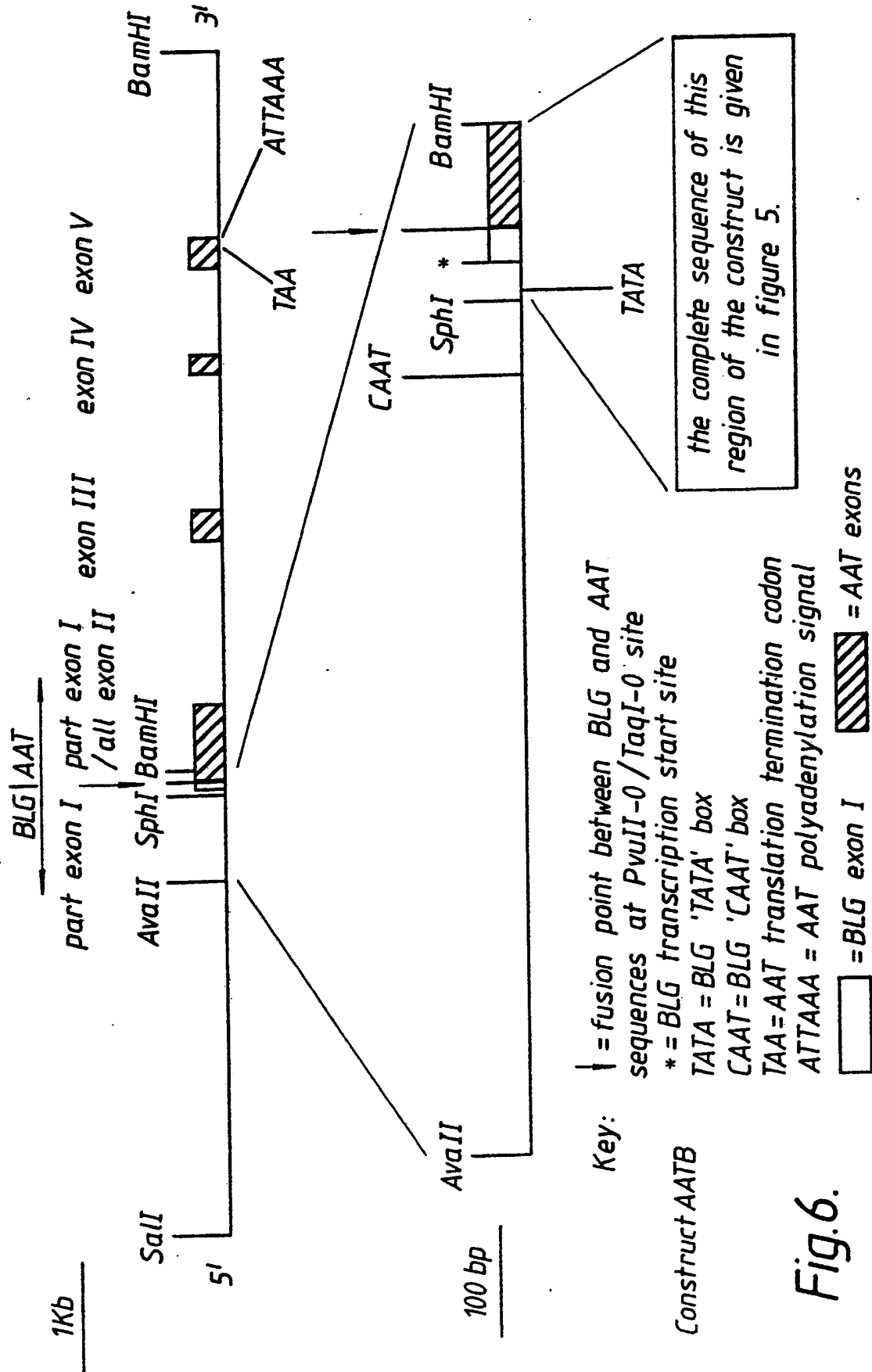
BLG = β -lactoglobulin

AAT = α ₁-antitrypsin

^^^ = indicate three nucleotides missing from the published sequence of Ciliberto, Dente & Cortese (1985) Cell 41, 531-540, but clearly present in the clone p8 α 1ppg procured from these authors. The nucleotides are present in the published sequence of α ₁-antitrypsin described by Long, Chandra, Woo, Davie & Kurachi (1984) Biochemistry 23, 4828-4837.

Fig. 5.

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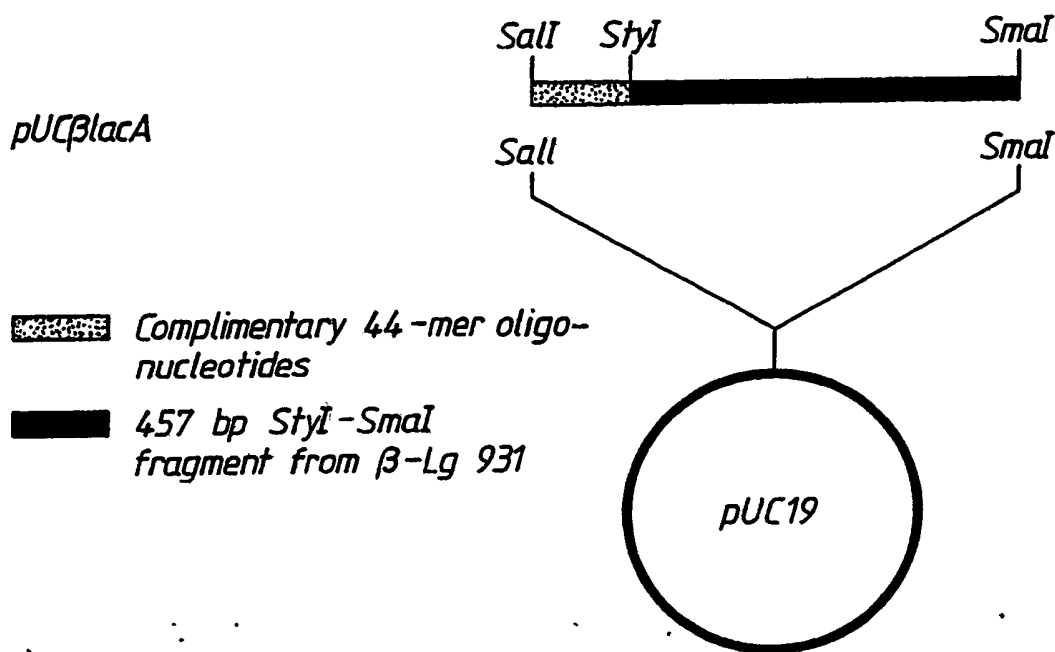
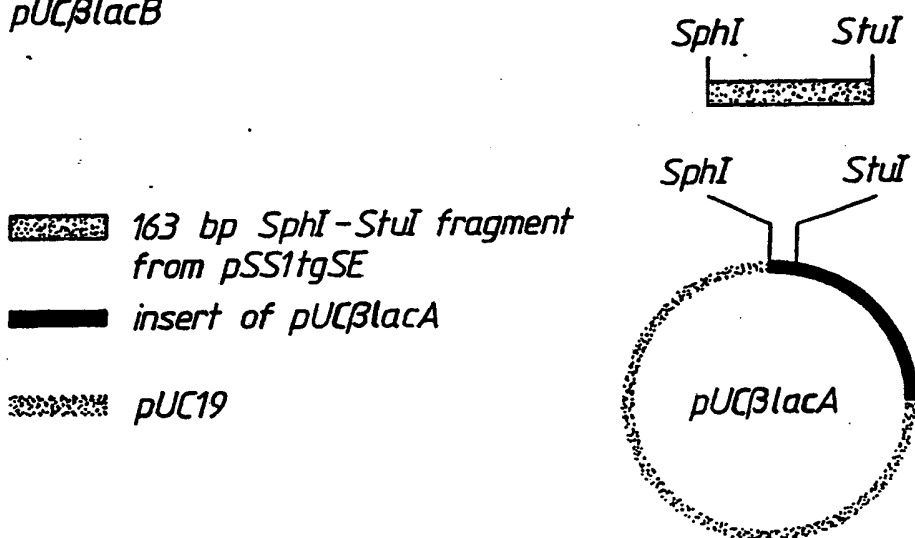
Construction of *pSS1tgXSΔClaBLG(BB)**pUCβlacB*

Fig. 7.

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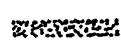
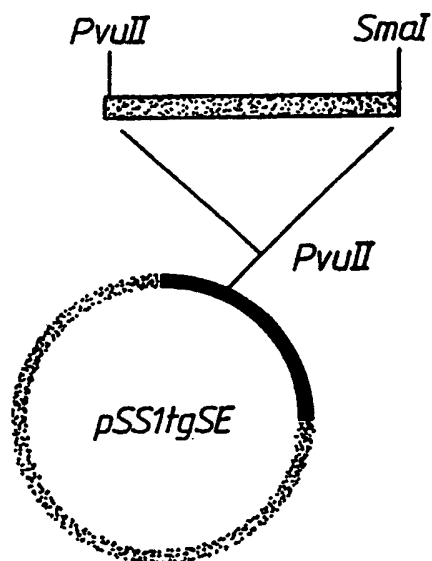
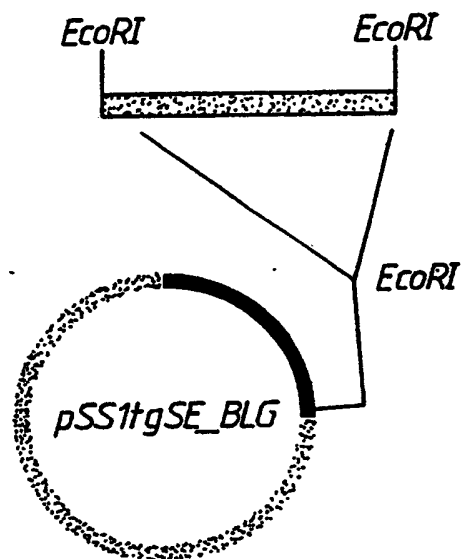


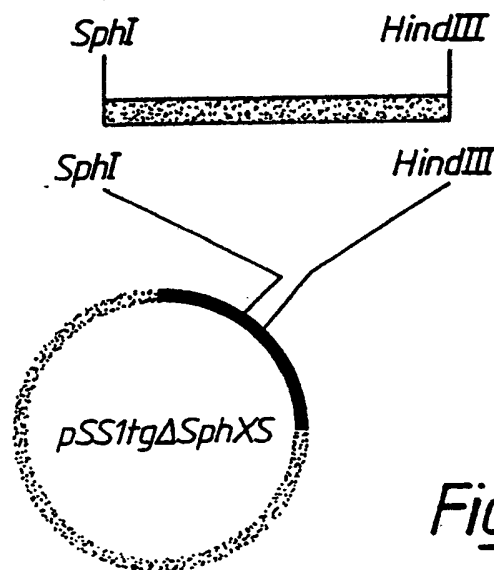
pSS1tgSE_BLG
 *ppoly*
 insert of *pSS1tgSE*
*pSE_BLG_3'*
 5.3 *EcoRI* partial fragment from *pSS1tgXSΔCla*
 *ppoly*
 insert
*pSS1tgXSΔClaBLG*
 3 kb *SphI*-*HindIII* fragment from *pSE_BLG_3'*
 insert of *pSS1tgΔSphXS*
 *ppoly*


Fig. 8.

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Construction of AATC:pSS1pUCXSAAT.TGA

1. Synthesis of oligonucleotides: 5' CTTGTGATATCG
3' CACTATAGCTTAA 5'

2. Ligate annealed oligos into *Sst*I/*Eco*RI cleaved pSS1tgSE to construct plasmid pSS1tgSE.TGA

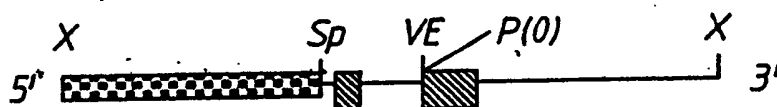


3. Cleave with *Eco*RI: Blunt with Klenow polymerase. Second cleavage with *Sph*I. Isolate *Sph*I-*Eco*RI (blunted) fragment.

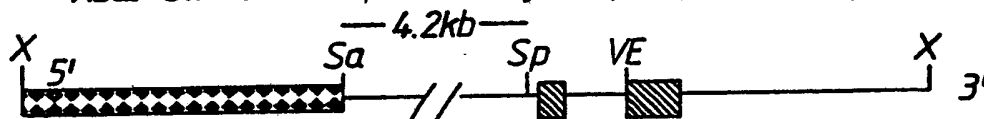


4. Cleave plasmid pBJ7 (this patent) with *Sph*I and *Pvu*II. Isolate large 4.3 kb) fragment.

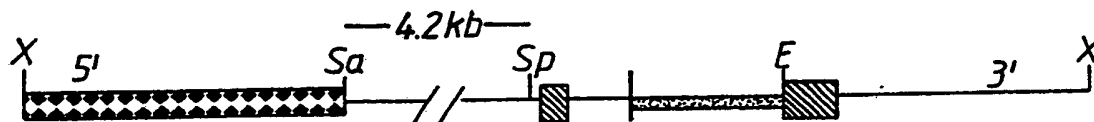
5. Ligate *Sph*I-*Eco*RI (blunt) fragment (3) with *Sph*I-*Pvu*II fragment (4) to produce pSS1tgSpX.TGA



6. Isolate *Sph*I-*Xba*I insert from pSS1tgSpX.TGA (5) and ligate to 4.2 kb *Sal*I-*Sph*I fragment from pSS1tgXS (previous patent) and *Xba*I-*Sal*I cleaved pUC18 to yield pSS1pUCXS.TGA



7. Insert *Acc*I-*Hind*III AAT insert from pUC8a1AT.73 (this patent) into the unique *Eco*RV site of pSS1pUCXS.TGA to produce pSS1pUCXSAAT.TGA. For microinjection the *Xba*I-*Sal*I fragment is excised from the vector.



■ pPOLY; ■ pUC18; — BLG intron or flanking,

■ BLG exons, ■ AAT, | oligo.

E, *Eco*RI; X, *Xba*I; Sa, *Sal*I; Sp, *Sph*I; V, *Eco*RV; St, *Sst*I; P(0), inactivated *Pvu*II site.

Fig. 9.

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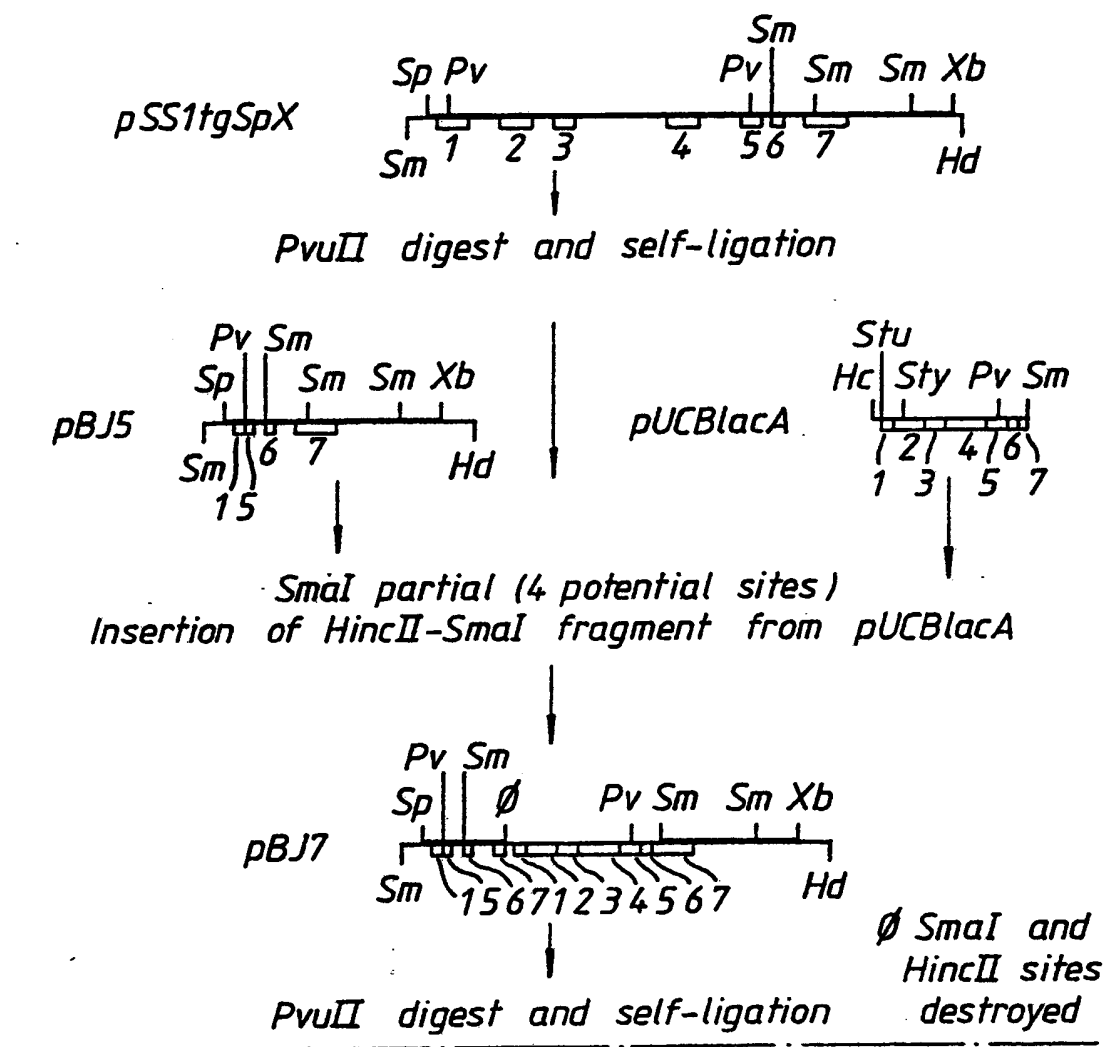


Fig.10a.

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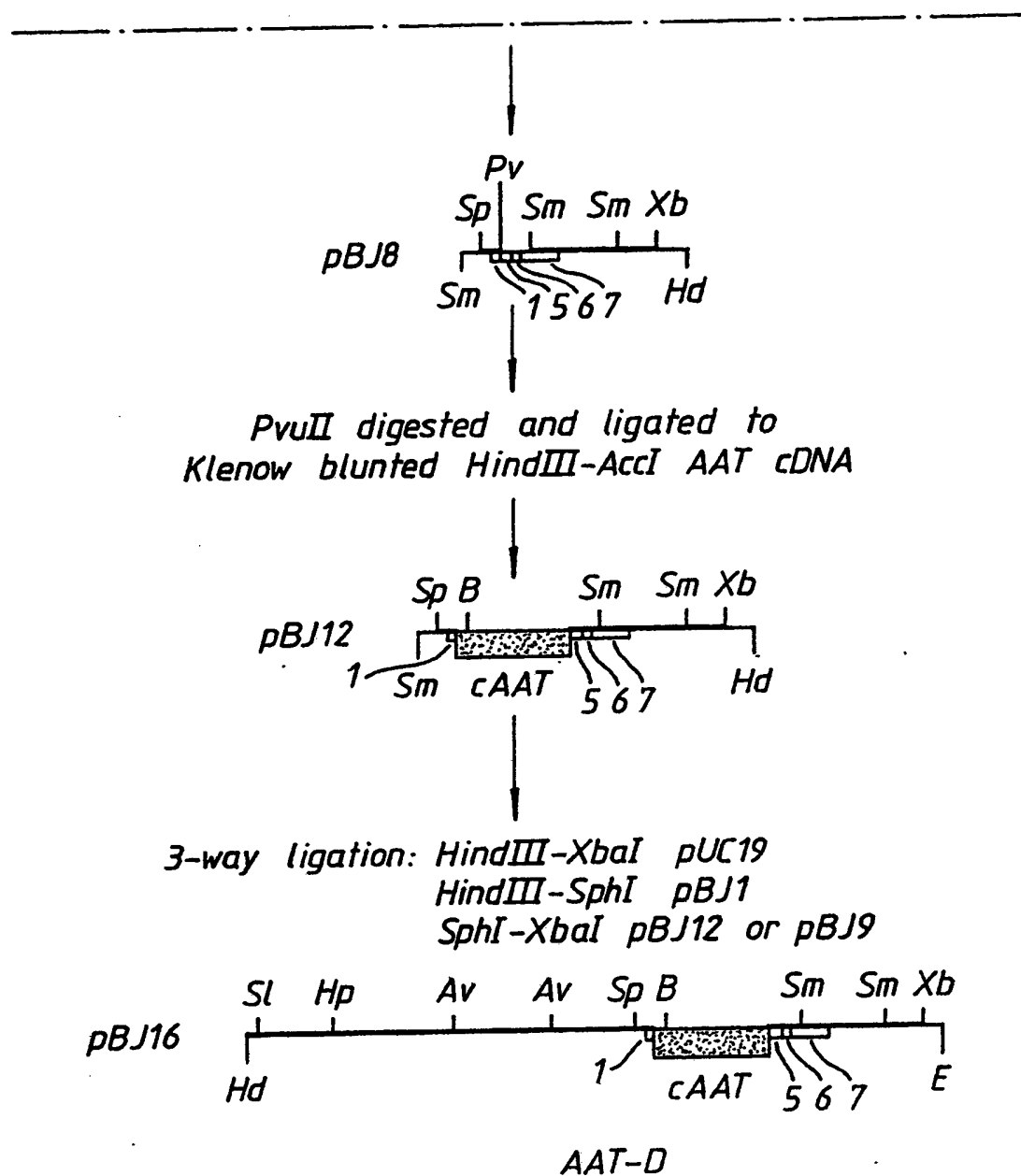
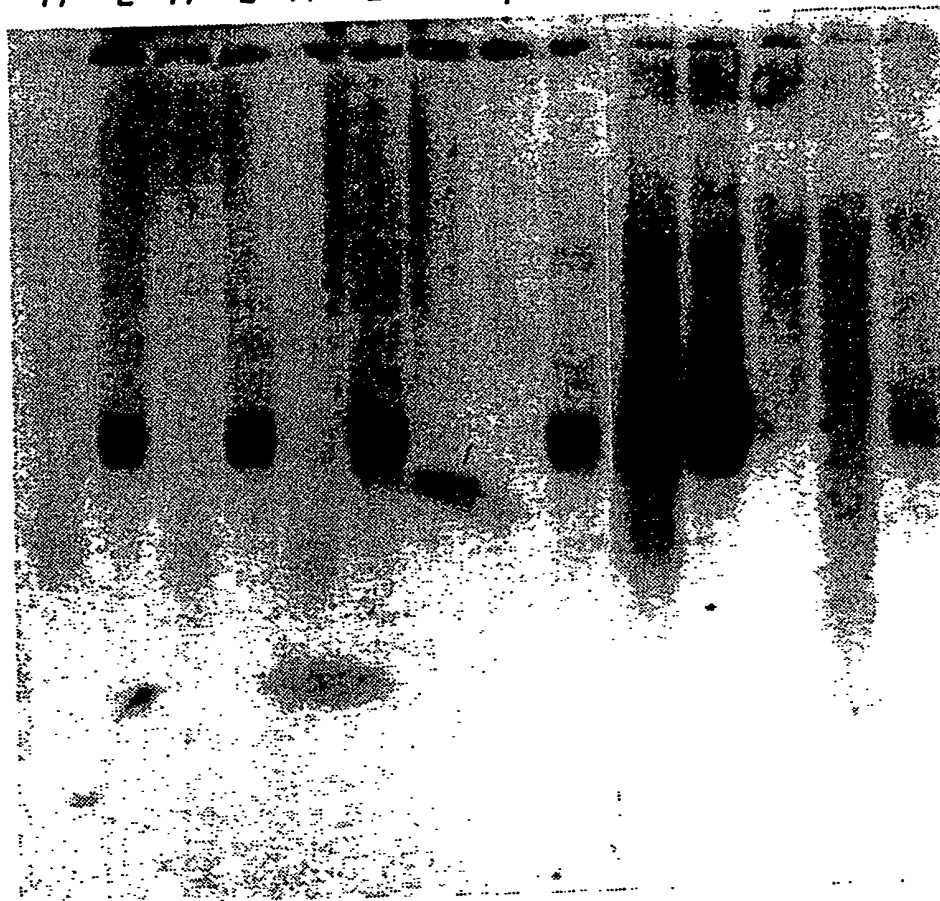


Fig.10b.

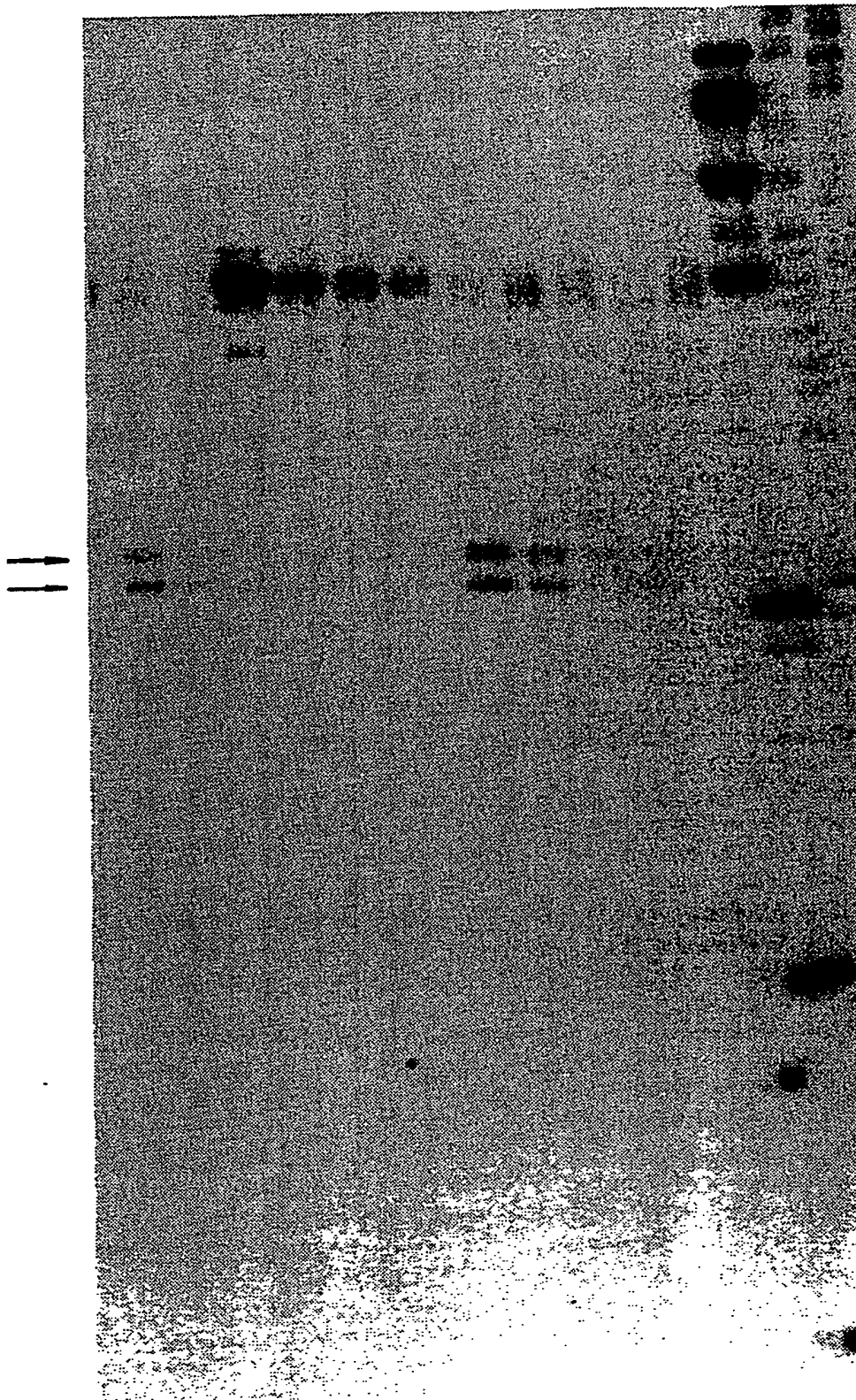
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1 2 3 4 5 6 7 8 9 10 11 12 13 14
M L M L M L K Sp Sa M L K Sp Sa

*Fig.11.*

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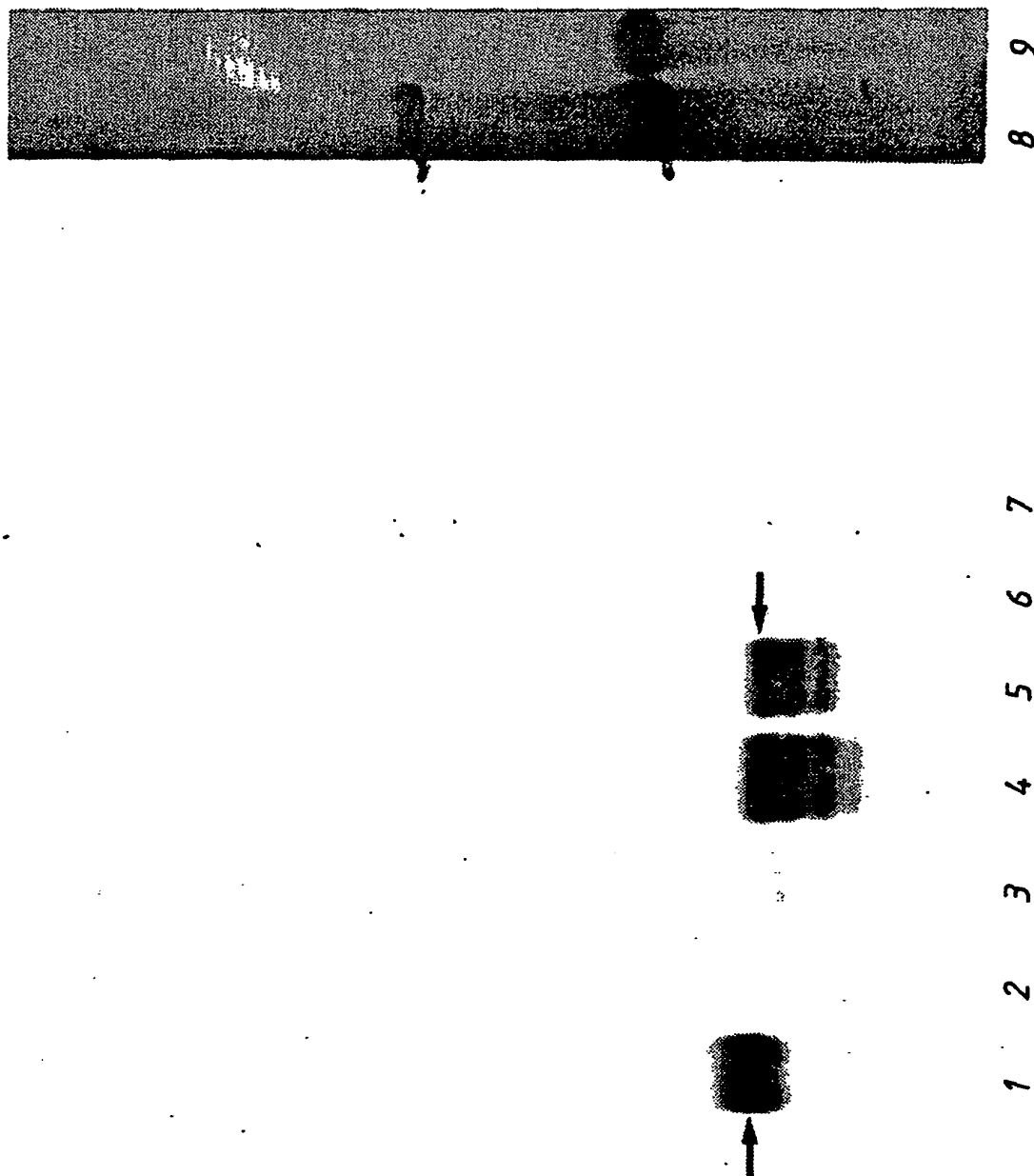
1 2 3 4 5 6 7 8 9 10 11 M

*Fig.12.*

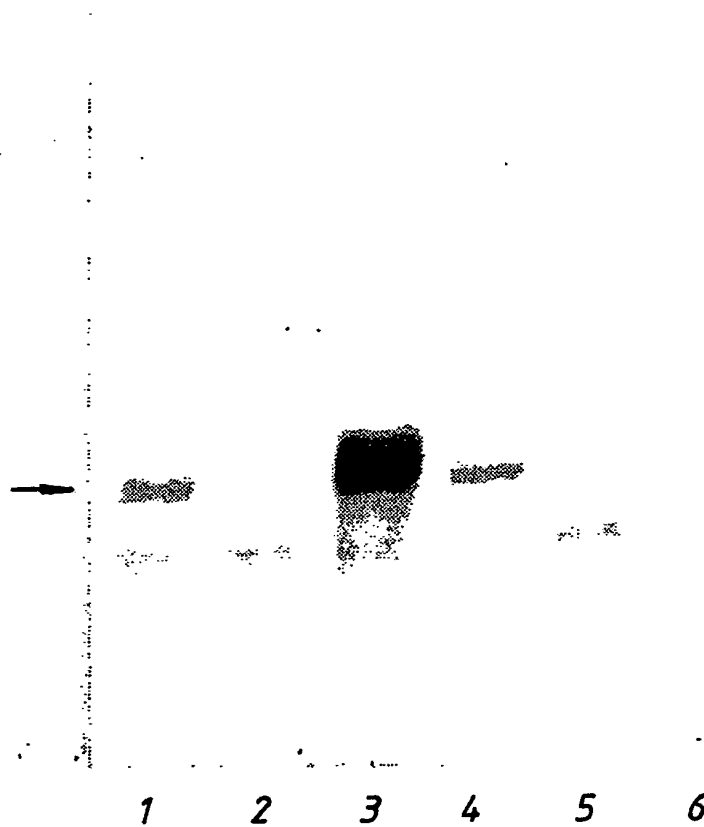
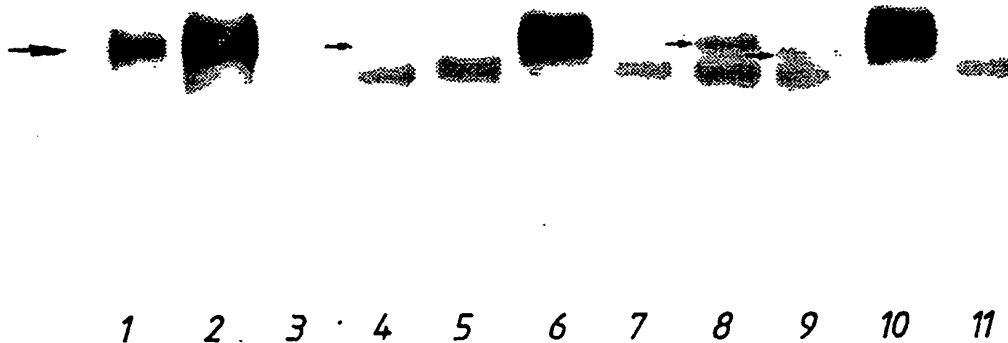
SUBSTITUTE SHEET

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Fig.13.



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EXPRESSION OF HUMAN AAT IN TRANSGENIC SHEEP MILK*Fig.14.**EXPRESSION OF HUMAN AAT IN THE MILK OF TRANSGENIC MICE**Fig.15.*

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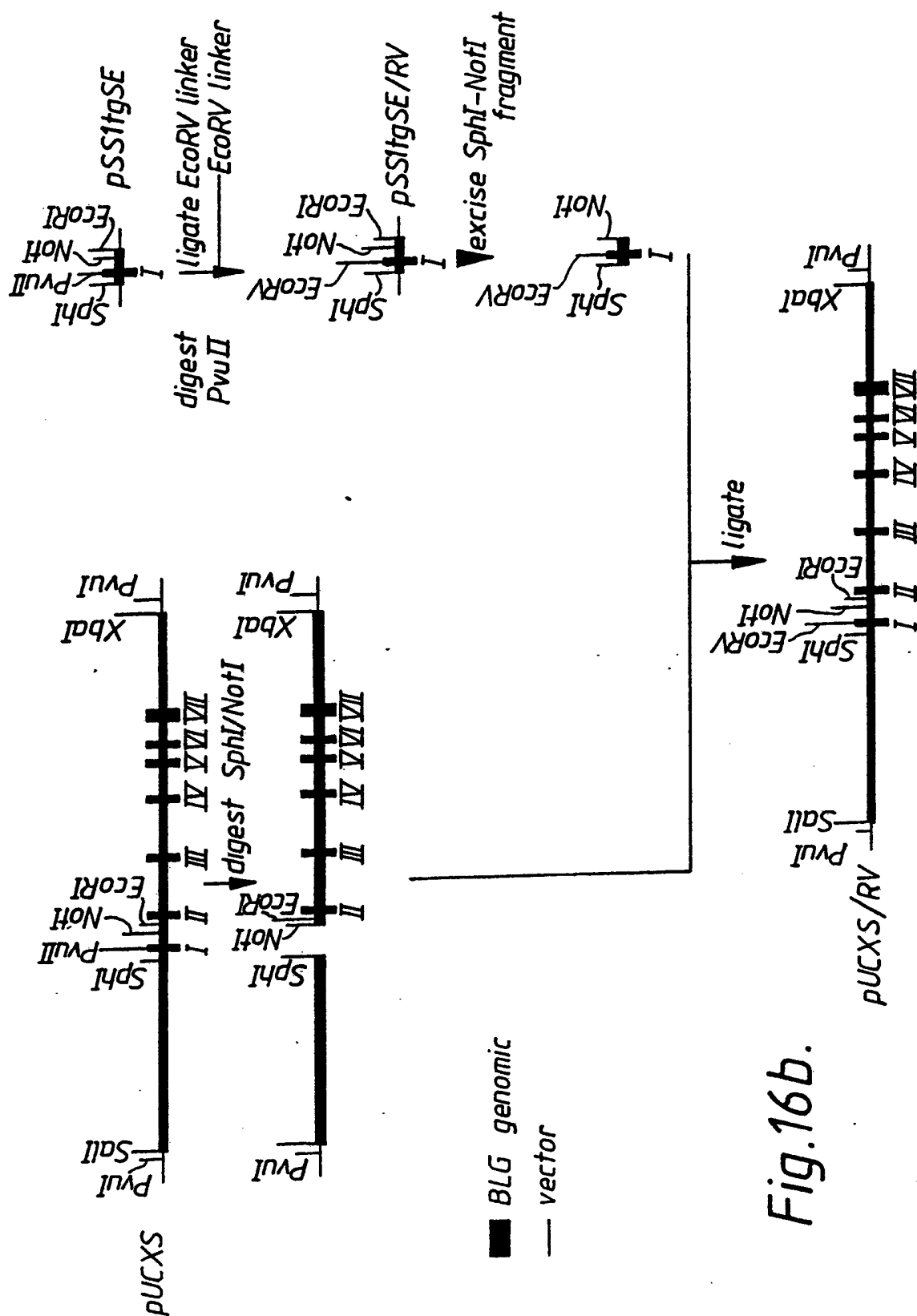


Fig.16b.

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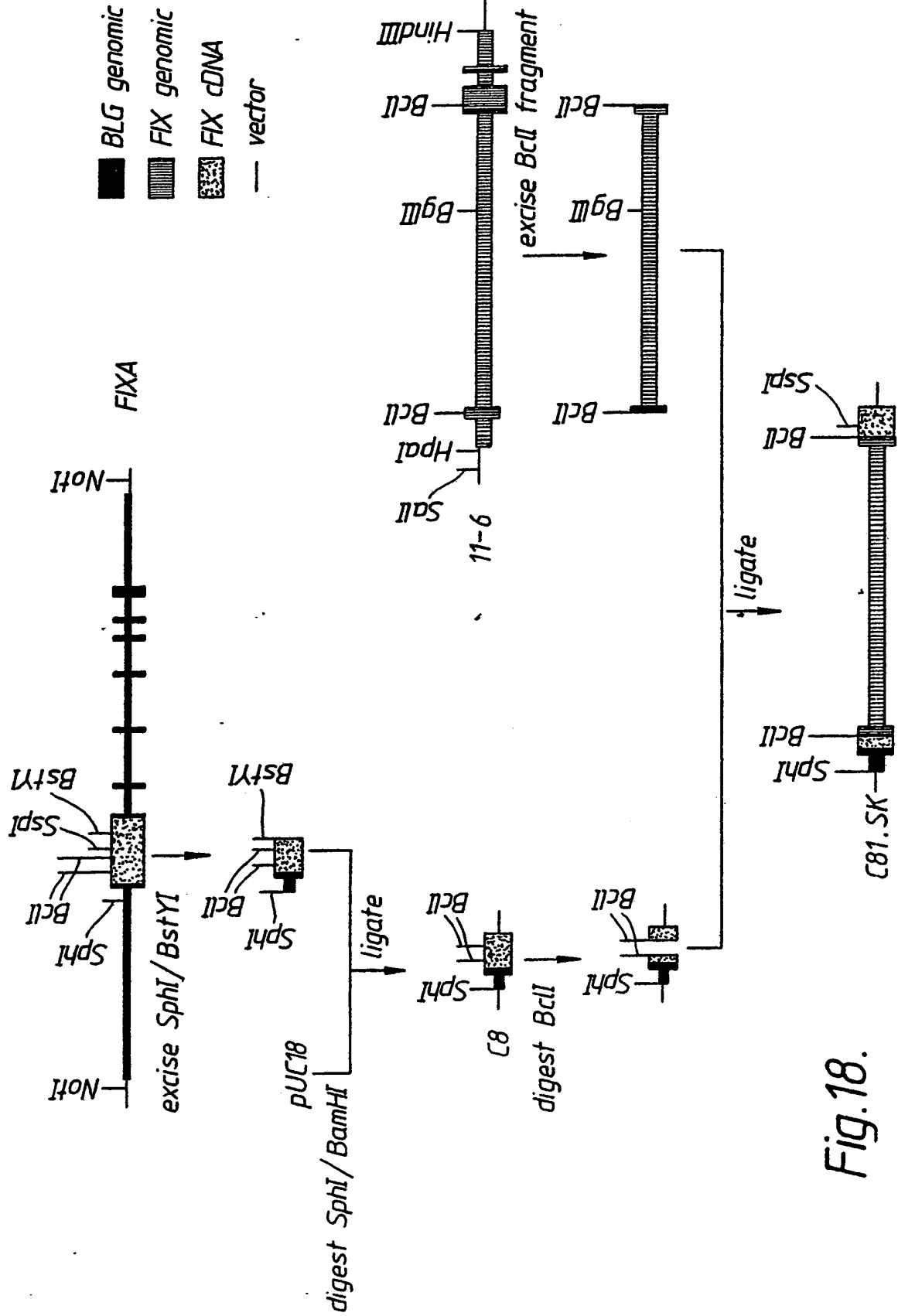


Fig.18.

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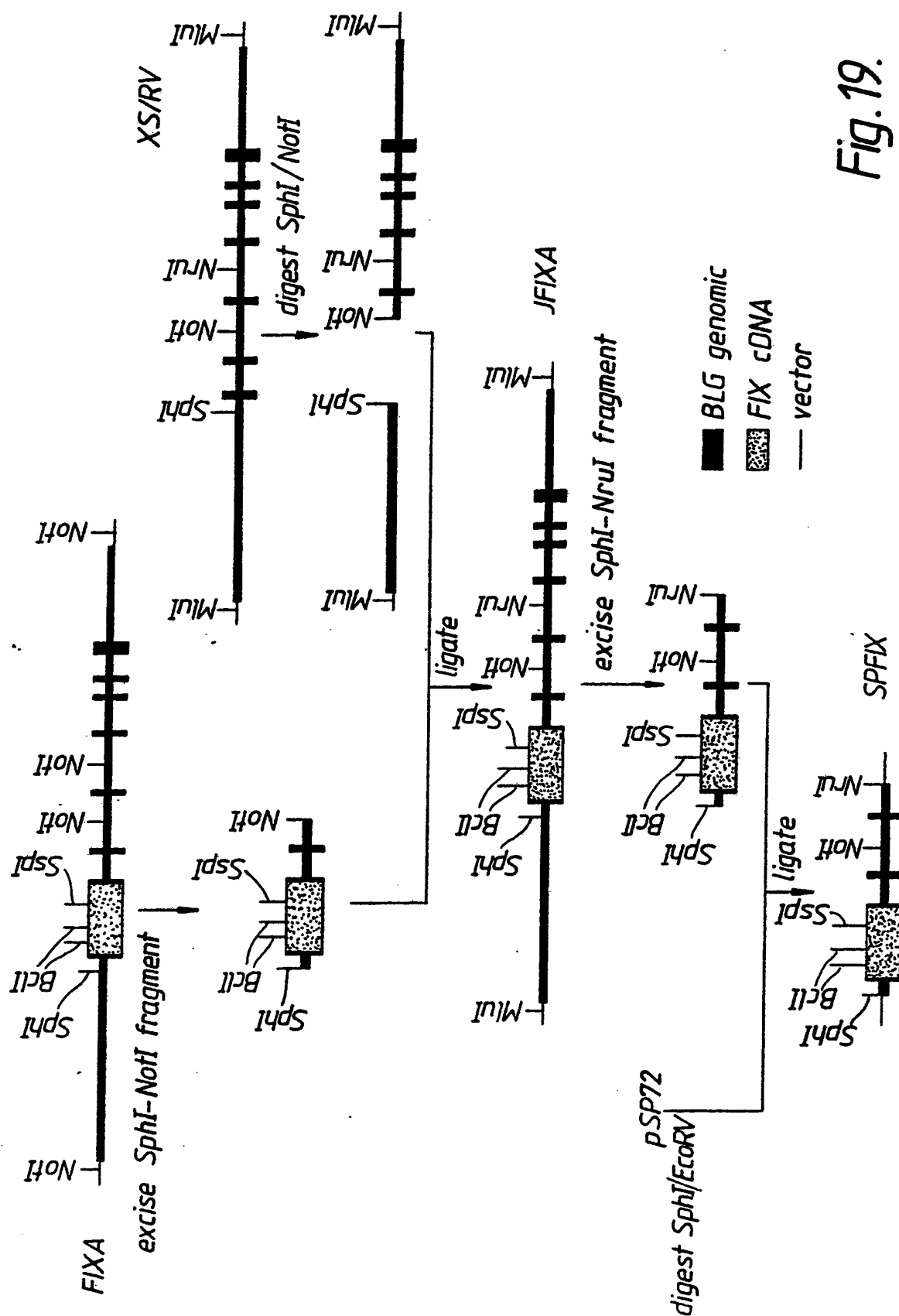


Fig. 19.

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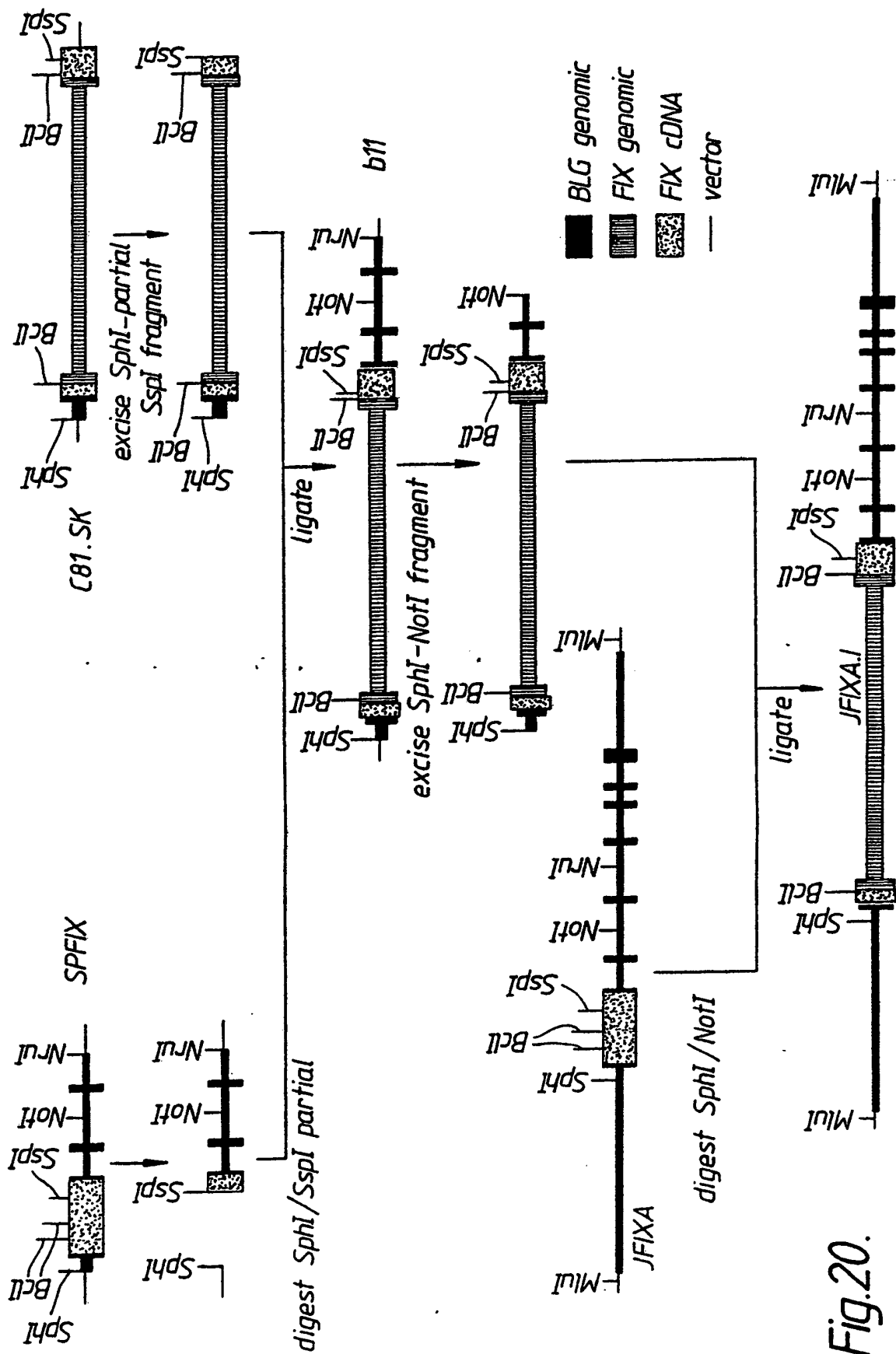


Fig. 20.

INTERNATIONAL SEARCH REPORT

International Application No **PCT/GB 89/01343**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 15/85, C 12 N 15/57		
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched †</div> <div style="display: flex; justify-content: space-between;"> Classification System : Classification Symbols </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> IPC5 C 12 N </div> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ‡</div>		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ‡		
Category *	Citation of Document, †† with indication, where appropriate, of the relevant passages ‡‡	Relevant to Claim No. ‡‡
Y	Proc. Natl. Acad. Sci., Vol. 85, 1988, (USA) Ralph L. Brinster et al: "Introns increase transcriptional efficiency in transgenic mice", see page 836 - page 840 --	1-17
Y	WO, A1, 88/00239 (PHARMACEUTICAL PROTEINS LTD) 14 January 1988, see page 19, line 10 - line 20; claim 20 --	1-17
Y	EP, A1, 0264166 (INTEGRATED GENETICS, INC.) 20 April 1988, see the whole document --	1-17
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ‡‡</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 24th January 1990		Date of Mailing of this International Search Report <div style="text-align: right;">24.1.90</div>
International Searching Authority <div style="text-align: center; margin-top: 10px;">EUROPEAN PATENT OFFICE</div>		Signature of Authorized Officer <div style="text-align: right; margin-top: 10px;">T.K. WILLIS</div>

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P,A Chemical Abstracts, volume 110, no. 19, 8 1
 May 1989, (Columbus, Ohio, US), Deng,
 Tiliang et al.: "Thymidylate synthase
 gene expression is stimulated by some
 (but not all) introns", see page 199,
 abstract 167168n, & Nucleic Acids Res
 1989, 17 (2), 645- 58
 --

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 15, 16 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(ii)

Plant or animal varieties or essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This international Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 89/01343**

SA 32133

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 08/11/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 88/00239	14/01/88	AU-D- 76490/87	29/01/88
		EP-A- 0274489	20/07/88
		JP-T- 1500162	26/01/89

EP-A1- 0264166	20/04/88	JP-A- 63000291	05/01/88

EPO FORM P079

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82